Remote Ischaemic Preconditioning

In Humans

Thesis presented for the degree of Doctor of Philosophy

in the Faculty of Medicine, University of London

Stavros Loukogeorgakis

Vascular Physiology Unit, Institute of Child Health

University College London

34 Great Ormond Street, London

WC1N 3JE, UK

Tel: +44 20 7813 8223

Fax: +44 20 7813 8532

Email: s.loukogeorgakis@ich.ucl.ac.uk

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Ischaemia-reperfusion (IR) injury contributes to tissue damage that occurs in myocardial infarction and stroke, and limits the outcome of current reperfusion strategies. Ischaemic preconditioning (IPC) is an innate mechanism that protects tissues from injury during ischaemia and subsequent reperfusion. IPC has systemic effects that protect tissues remote for those undergoing preconditioning (remote IPC; RIPC). RIPC might enable the clinical utility of ischaemic preconditioning to be tested, as protection of vital tissues against IR injury could be achieved by remotely preconditioning a non-vital tissue. This thesis sought to determine the optimal conditions for inducing RIPC in humans.

The protective effects of RIPC were investigated in healthy volunteers, using an *in vivo* model of IR injury to the vascular endothelium of the brachial artery. RIPC of the limb protected against IR-induced endothelial dysfunction and the degree of protection depended on the characteristics of the preconditioning stimulus. Two phases of protection by RIPC were evident; an early relatively short phase, active immediately, and a second window of protection, which is more prolonged lasting for up to 48 hours following the application of the RIPC stimulus. Repeated application of the RIPC stimulus caused tissue protection for up to 7 days. RIPC was shown to be dependent on intact autonomic function and the opening of ATP-sensitive potassium channels. The protective effects of RIPC were investigated in paediatric patients undergoing renal transplantation. Data from this clinical study are consistent with a beneficial effect of RIPC against IR injury to the renal graft and suggest that RIPC may have therapeutic potential in clinical IR syndromes.

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Chapter 1:

General Introduction

1.1 Introduction

Ischaemia of tissues such as the myocardium and brain, due to thrombosis-induced arterial occlusion, is a major clinical manifestation of atherosclerosis and cause of morbidity and mortality in the economically developed world. Tissue ischaemia initially leads to cellular metabolic dysfunction and if prolonged causes cell death. Reperfusion is essential to protecting ischaemic tissues (225), and such therapies have become routinely available in clinical practice (1;183). However, reperfusion of an ischaemic area may result in reperfusion injury so that the resultant cellular damage is a composite of that occurring during ischaemia and reperfusion.

These facets of ischaemia reperfusion also apply to the blood vessels themselves. The vascular endothelium is susceptible to ischaemia-reperfusion injury (431). Endothelial integrity is necessary for adequate vasomotor reactivity of the coronary circulation, which is not only vital for the regulation of the blood supply to the heart under normal physiological conditions, but is also for reperfusion following ischaemia. Furthermore, the vascular endothelium plays a key role in the control of both leukocyte and platelet function and in the modulation of inflammatory and thrombotic events associated with ischaemia-reperfusion injury and myocardial infarction.

One strategy that shows promise to reduce ischaemia-reperfusion injury is ischaemic preconditioning, an innate mechanism that protects tissues from injury during ischaemia and subsequent reperfusion (538). Organs can be preconditioned by brief, non-lethal periods of ischaemia, and are subsequently able to withstand the effects of prolonged episodes of future ischaemia. Ischaemic preconditioning also has systemic effects to protect tissues that are distant or remote from the tissue on which the

preconditioning stimulus is applied (399). This aspect of preconditioning is termed remote ischaemic preconditioning (RIPC), has been demonstrated in many different species to protect vital organs and other tissues from ischaemia-reperfusion injury. The magnitude of this protection approaches that of local ischaemic preconditioning, certainly in the context of myocardial infarction. Moreover, RIPC is induced, non-invasively, by short periods of ischaemia and reperfusion of non-vital tissues, including the limb (243). This might enable the protective effects of ischaemic preconditioning to be harnessed in human cardiovascular disease.

This thesis will present experimental data from human vascular studies that investigate the potential of remote ischaemic preconditioning to protect against ischaemia-reperfusion injury.

1.2 Ischaemia-reperfusion injury

1.2.1 Epidemiology of the atherosclerotic vascular occlusion

Atherosclerosis is characterised by the deposition of atheromatous plaques that narrow the arterial lumen. It has a long pre-clinical phase, with atherosclerotic lesions developing at a young age. Multiple factors affect the progression of the disease throughout adult life and the clinical complications of atherosclerosis are mainly a disease of middle age. Arterial narrowing predisposes to thrombosis and acute arterial occlusion depriving distal tissues of their blood supply, resulting in myocardial infarction and stroke when those tissues are heart and brain respectively. Atherosclerosis and its ischaemic complications affecting the heart and the brain are the most common cause of death in the western world (86;374) and is also an increasing health problem in developing countries (86;345). Ischaemic

complications of atherosclerosis are responsible for more than fifty percent of the annual mortality in the United Kingdom. There are 110,000 deaths in the UK from ischaemic heart disease each year. Stroke is the biggest cause of severe disability and the third most common cause of death in the United Kingdom.

1.2.2 Pathophysiology of ischaemia-reperfusion injury

These mechanisms have been best characterised in the myocardium but are thought to be representative of processes in other tissues.

1.2.2.1 Ischaemic injury

The myocardium can tolerate brief periods (up to approximately 15 minutes) of total ischaemia without resulting in cardiomyocyte death (250;251). Damage caused by such brief periods of ischaemia is usually reversible with prompt arterial reperfusion. However, in the clinical setting of myocardial infarction, coronary artery bypass graft (CABG) surgery or cardiac transplantation, myocytes suffer longer and more severe periods of ischaemia that have complex effects.

1.2.2.1.1 Effect of ischaemia on myocardial metabolic state

During the first minutes of ischaemia, the production of high-energy phosphates (sum of ATP and phosphocreatine) declines and it is greatly exceeded by utilisation by the myocardium (224). Therefore, there is a rapid reduction in the myocardial stores of ATP and phosphocreatine (PCr), with PCr levels falling more rapidly than ATP levels *(figure 1.1)* (490). PCr is depleted by transferring the high-energy phosphate group it contains to ADP in an attempt to maintain cellular ATP levels. In the presence of normal aerobic conditions, energy production occurs in the mitochondria by oxidative

phosphorylation using fatty acids as the main substrate, or by carbohydrate metabolism via the tricarboxylic acid (Krebs) cycle (47;463). In the absence of normal oxidative phosphorylation, ATP is converted to AMP. This is broken down via adenosine, inosine and hypoxanthine to xanthine (226). When intracellular ATP concentration is reduced below 20% of normal values, the cardiomyocyte loses capacity to produce high-energy phosphates, preserve cell volume and maintain ionic regulation (453). The resultant is *irreversible* cell damage leading to myocardial necrosis during ischaemia (and subsequent reperfusion; *see section 1.2.2.2.1*).

Under normal aerobic conditions, myocardial energy requirements are mostly met by oxidation of free fatty acids (FFA), in the form of fatty (acyl) esters containing co-enzyme A (acyl-CoA) (47). Acyl-CoA is released by the breakdown of acyl esters and is normally transported from the cytoplasm to the mitochondria (via the acyl-CoA-carnitine transferase system) where it is utilised for the generation of ATP. Myocardial ischaemia inhibits fatty acid oxidation *(figure 1.1)*, which combined with reduced acyl-CoA-carnitine transferase activity, increases concentrations of acyl-CoA in the cytoplasm (523). Accumulation of acyl-CoA may be deleterious because it inhibits formation of fatty acid acyl esters and adenine nucleotide translocase (ANT). ANT transports ATP synthesised in the mitochondria to the cytoplasm; reduced ANT activity and failure to replete cytoplasmic ATP may contribute to the decline of PCr in the ischaemic myocardium (204;205). Acyl-CoA can also cause direct damage to cardiomyocytes by acting as detergents to disrupt the sarcolemmal membrane.

The ischaemic myocardium utilises glucose to generate ATP through glycolysis. Indeed, augmentation of the glycolytic flux by provision of glucose or prior

augmentation of glycogen stores confers some resistance to ischaemia (205;252). However, when ischaemia and hypoxia persist, energy production switches to anaerobic pathways. Anaerobic glycolysis alone cannot maintain myocardial ATP stores indefinitely *(figure 1.1)*, and is associated with accumulation of lactate, due to the inhibition of the Krebs cycle and the lack of metabolite washout because of the lack of perfusion (354). This may be one of the most important characteristics of ischaemia and the main difference between ischaemia and hypoxia. In hypoxic conditions, there is lack of oxygen, but adequate perfusion is maintained and as a result by-products of anaerobic metabolism can be removed. However, in the setting of myocardial infarction, perfusion of the infarcted area ceases and in addition to the lack of oxygen, there is increased concentration of anaerobic metabolites such as lactate. This results in reduced intracellular pH and is the cause of intracellular acidosis observed during ischaemia (113;454). Decreased intracellular pH and increased lactate (and NADH) concentration (together with lack of oxygen) inhibit the glycolytic flux that is augmented during the initial stages of ischaemia (414).

The disruption of the energy balance in cardiomyocytes (induced by depletion of ATP stores) initiates a cascade of events that, if not reversed by timely reperfusion, will ultimately lead to cellular necrosis. During the initial stages of ischaemia, there is a continuous reduction in the cytoplasmic concentration of ATP. In the heart, this leads to *rigor contracture* in the ischaemic myocardium (394). Following ATP depletion (prolonged ischaemia) the myocardium remains in a contracted state as all cross-bridges formed between actin and myosin in cardiomyocytes remain in an attached state (357;395). The contracture developed by this ischaemic mechanism does not cause major cellular damage but results in cytoskeletal defects that render the

cardiomyocytes more fragile and thus more susceptible to swelling-induced destruction observed during ischaemia (and more importantly on reperfusion) (422).

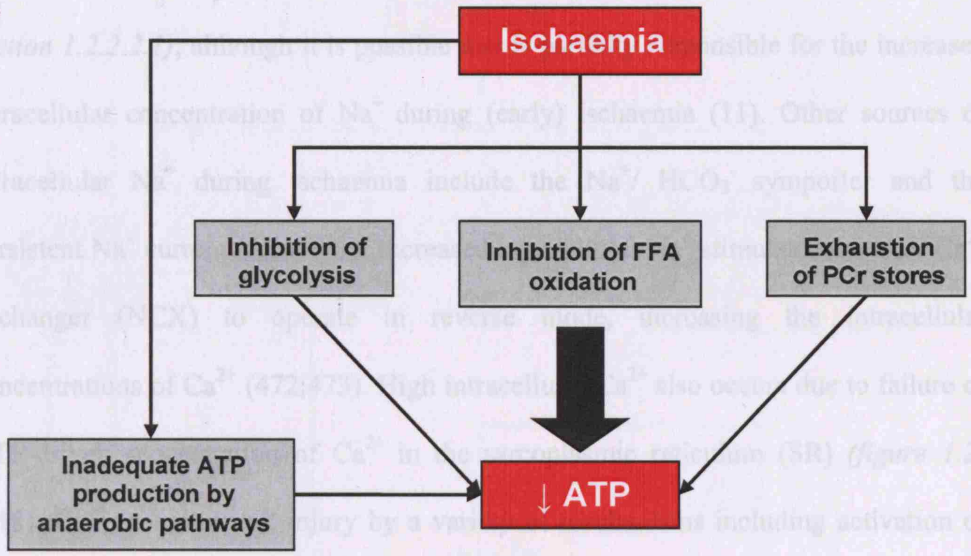


Figure 1.1 Metabolic effects of myocardial ischaemia. Adapted from Jennings *et al*, Am J Pathol; 102: 241, 1981. *FFA: free fatty acids; PCr: phosphocreatine.*

1.2.2.1.2 Effect of ischaemia on myocardial osmotic and ionic balance

Another direct result of ATP depletion is the inactivation of the Na^+/K^+ pump *(figure 1.2)* (226). This increases intracellular Na^+ concentration (with concomitant increase of extracellular K^+ concentrations) and disrupts the sarcolemmal membrane potential (99). Na^+ also enters cardiomyocytes as a result of intracellular acidosis. Increased intracellular H^+ concentration stimulates acid extrusion via the cardiac Na^+/ H^+ exchanger (NHE1) (496), leading to the entry of a Na^+ ion for each H^+ removed. It has been suggested that NHE1 is initially active during ischaemia but as protons are extruded from myocardial cells, they accumulate in the extracellular space (due to lack of perfusion) and eventually inhibit further activity (284). Recent evidence also

suggests that NHE1 inhibition could also be due to depletion of ATP (81;167), despite the fact that NHE1 is a classical exchanger and does not require metabolic energy for its operation (10). It is likely that NHE1 mainly operates during reperfusion *(see section 1.2.2.2.1)*, although it is possible that is partially responsible for the increased intracellular concentration of Na^+ during (early) ischaemia (11). Other sources of intracellular Na^+ during ischaemia include the Na^+/ HCO_3^- symporter and the persistent Na^+ current ($I_{Na,P}$) (11). Increased intracellular Na^+ stimulates the Na^+/ Ca^{2+} exchanger (NCX) to operate in reverse mode, increasing the intracellular concentrations of Ca^{2+} (472;473). High intracellular Ca^{2+} also occurs due to failure of ATP-driven sequestration of Ca^{2+} in the sarcoplasmic reticulum (SR) *(figure 1.2)* (218). Ca^{2+} promotes cell injury by a variety of mechanisms including activation of proteases, phospholipases and nucleases *(figure 1.3)* (312;541).

Osmotic imbalance between the intracellular and extracellular compartments, induced by increased intracellular Na^+, H^+, Ca^{2+}, as well as the osmotic pull of lactate, phosphate, adenine nucleotide degradation products and long chain fatty acids (from the breakdown of sarcolemmal phospholipids), may cause swelling of cardiomyocytes *(figure 1.3)* (404;453). Increased fragility and swelling causes cell rupture, but this is not common during ischaemia, as the accumulation of metabolites in the extracellular space (due to lack of perfusion) re-establishes the osmotic balance across the sarcolemmal membrane and prevents water from entering the cardiomyocytes. Catastrophic swelling is a more common feature of reperfusion injury *(see section 1.2.2.2.1)*.

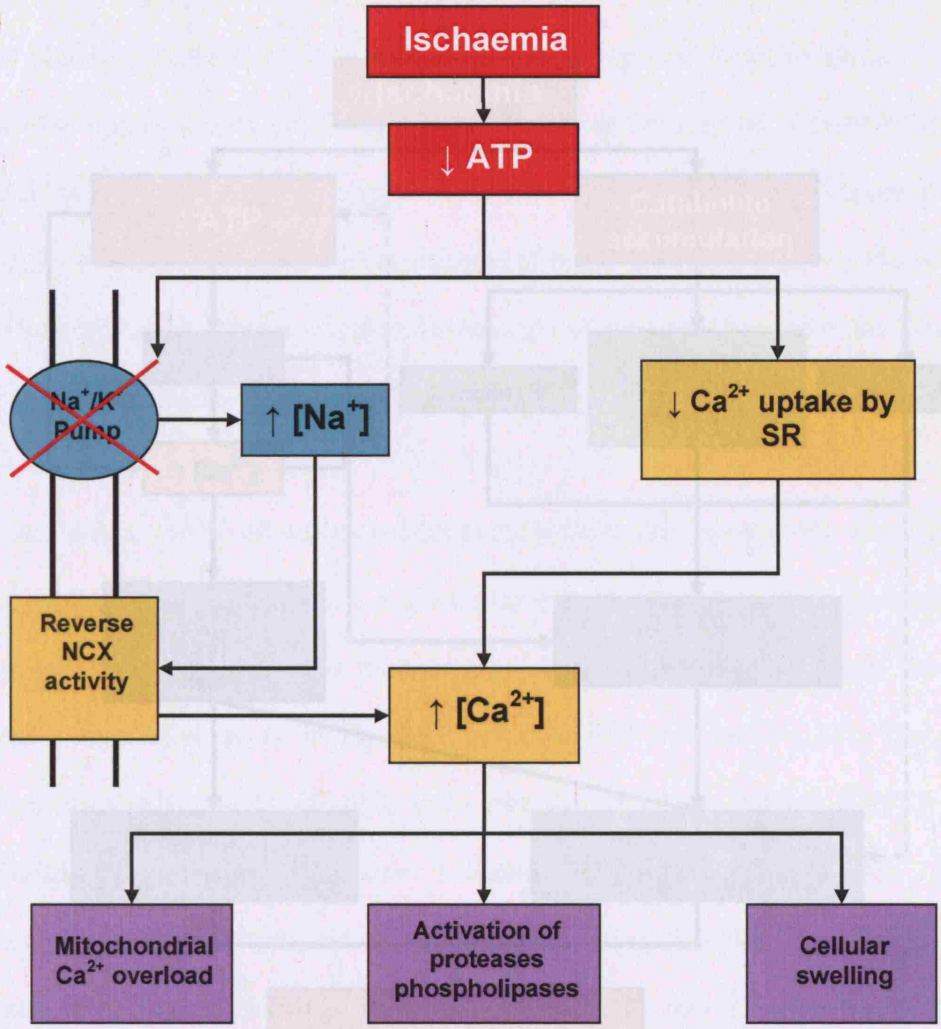


Figure 1.2 Interactions between ischaemia and intracellular Ca^{2+} concentration in the myocardium. Adapted from Braunwald *et al*, N Eng J Med; 307: 1618, 1982. *SR: sarcoplasmic reticulum; NCX: Na^+/Ca^+ exchanger.*

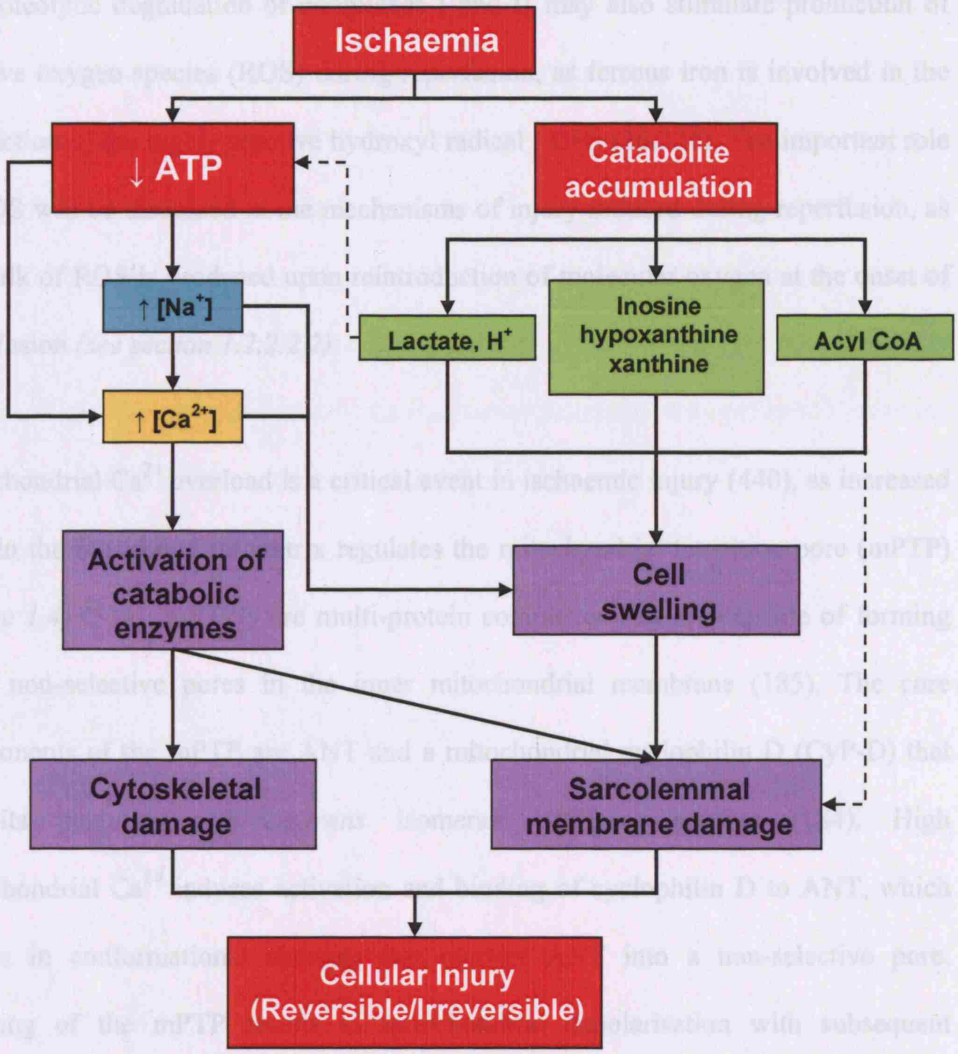


Figure 1.3 Pathways of cellular injury during ischaemia. For details see *sections 1.2.2.1.1* and *1.2.2.1.2*.

1.2.2.1.3 Effects of ischaemia on mitochondrial structure and function

The mitochondrial electron transport complexes are susceptible to ischaemic damage, particularly complexes I, III and V (ATP synthase) *(figure 1.4)* (413). The resulting electron leakage increases free electrons available for molecular oxygen reduction and

superoxide ($O_2^{-}\cdot$) production upon reperfusion (221). Liberation of ferrous iron (Fe^{2+}), by proteolytic degradation of complexes I and II may also stimulate production of reactive oxygen species (ROS) during reperfusion, as ferrous iron is involved in the production of the highly reactive hydroxyl radical (·OH) (26;221). The important role of ROS will be discussed in the mechanisms of injury induced during reperfusion, as the bulk of ROS is produced upon reintroduction of molecular oxygen at the onset of reperfusion *(see section 1.2.2.2.2)*.

Mitochondrial Ca^{2+} overload is a critical event in ischaemic injury (440), as increased Ca^{2+} in the mitochondrial matrix regulates the mitochondrial transition pore (mPTP) *(figure 1.4)* (514). mPTP's are multi-protein complexes that are capable of forming large non-selective pores in the inner mitochondrial membrane (185). The core components of the mPTP are ANT and a mitochondrial cyclophilin D (CyP-D) that exhibits peptidyl-prolyl *cis-trans* isomerase (PPIase) activity (184). High mitochondrial Ca^{2+} induces activation and binding of cyclophilin D to ANT, which results in conformational changes that convert ANT into a non-selective pore. Opening of the mPTP results in mitochondrial depolarisation with subsequent uncoupling of oxidative phosphorylation (184). Mitochondrial swelling is another consequence of mPTP opening. Increased inner membrane permeability allows solutes and water to enter the matrix and rupture the outer membrane. Such mitochondrial damage can result in cardiomyocyte death by necrosis as affected cells will be unable to re-establish energy production even when the oxygen supply is restored (184). In addition, outer membrane rupture results in the release of molecules residing in the inter-membrane space, including cytochrome c, Smac/DIABLO and others, which promote apoptotic cell death (312). Recent evidence suggests that

mPTP opening and associated mitochondrial damage do not occur during ischaemia (172). Despite conditions favouring mPTP opening (high Ca^{2+} and phosphate and low levels of adenine nucleotides), this is prevented by the high intracellular pH *(figure 1.4)*. During reperfusion intracellular pH is quickly restored to normal values and as a result mPTP opening occurs during reperfusion. However, ischaemia "primes" the mPTP for opening *(figure 1.4)*, and longer periods of ischaemia result in more generalised and prolonged mPTP opening upon reperfusion (184). Severe ischaemia can cause mitochondrial destruction via membrane degradation by phospholipases and proteases and thus lead to cellular necrosis *(figure 1.4)*.

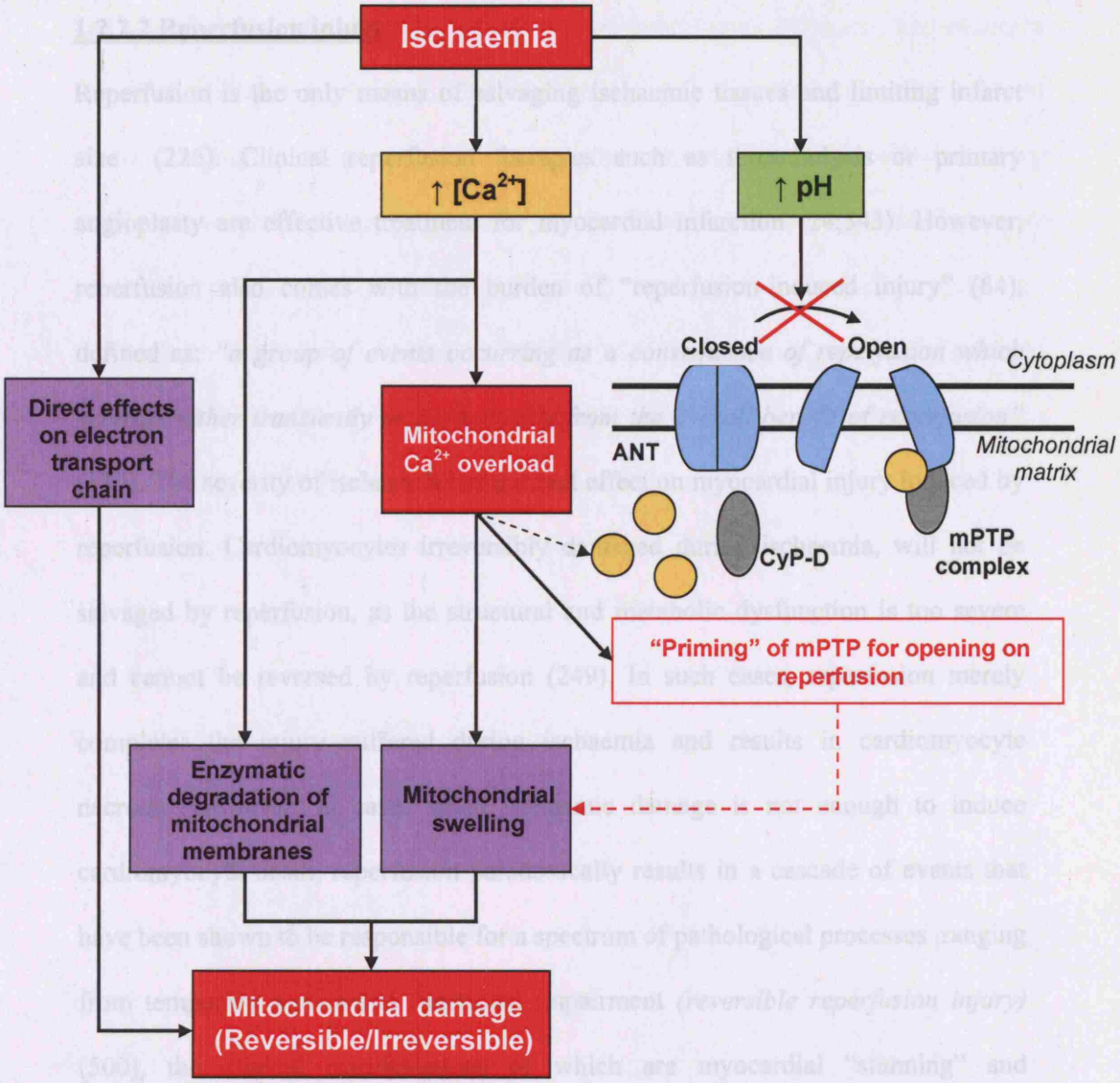


Figure 1.4 Effects of ischaemia on mitochondria. For details see *section 1.2.2.1.3*. Note that during ischaemia, high intracellular pH inhibits the opening of the mitochondrial permeability transition pore *(mPTP)*, but ischaemic conditions "prime" the pore for opening during reperfusion. *ANT: adenine nucleotide translocase; CyP-D:cyclophilin-D.*

1.2.2.2 Reperfusion injury

Reperfusion is the only means of salvaging ischaemic tissues and limiting infarct size (225). Clinical reperfusion therapies such as thrombolysis or primary angioplasty are effective treatment for myocardial infarction (24;543). However, reperfusion also comes with the burden of "reperfusion-induced injury" (64), defined as: *"a group of events occurring as a consequence of reperfusion which subtract either transiently or permanently from the overall benefit of reperfusion"* (519). The severity of ischaemia has a direct effect on myocardial injury induced by reperfusion. Cardiomyocytes irreversibly damaged during ischaemia, will not be salvaged by reperfusion, as the structural and metabolic dysfunction is too severe and cannot be reversed by reperfusion (249). In such cases, reperfusion merely completes the injury suffered during ischaemia and results in cardiomyocyte necrosis. However, in cases when ischaemic damage is not enough to induce cardiomyocyte death, reperfusion paradoxically results in a cascade of events that have been shown to be responsible for a spectrum of pathological processes ranging from temporary myocardial functional impairment *(reversible reperfusion injury)* (500), the clinical manifestations of which are myocardial "stunning" and reperfusion arrhythmias (56;63;200), to severe *(irreversible)* reperfusion injury with associated cardiomyocyte death via necrosis or apoptosis *(figure 1.5)* (13;132;326).

Reperfusion injury of the myocardium is a complex phenomenon, initiated immediately at the onset of reperfusion *(early reperfusion injury)* (394) and if left unchecked can continue for hours or days following reperfusion *(late reperfusion injury)* (239). Early reperfusion injury involves production of free radicals due to the re-introduction of molecular oxygen (15), and the disruption of the metabolic, osmotic

and ionic balance in myocardial cells that is initiated during ischaemia and becomes complete during reperfusion (227;453). During early reperfusion myocardial cell death is mainly due to necrosis (394). In contrast, late myocardial reperfusion injury involves myocardial damage induced by a complex inflammatory response (involving pro-inflammatory cytokines and neutrophils) that can lead to late myocardial functional impairment and/or necrosis (147;148), and also programmed cardiomyocyte death via apoptotic mechanisms (initiated at the onset of reperfusion and completed later) *(figure 1.5)* (168).

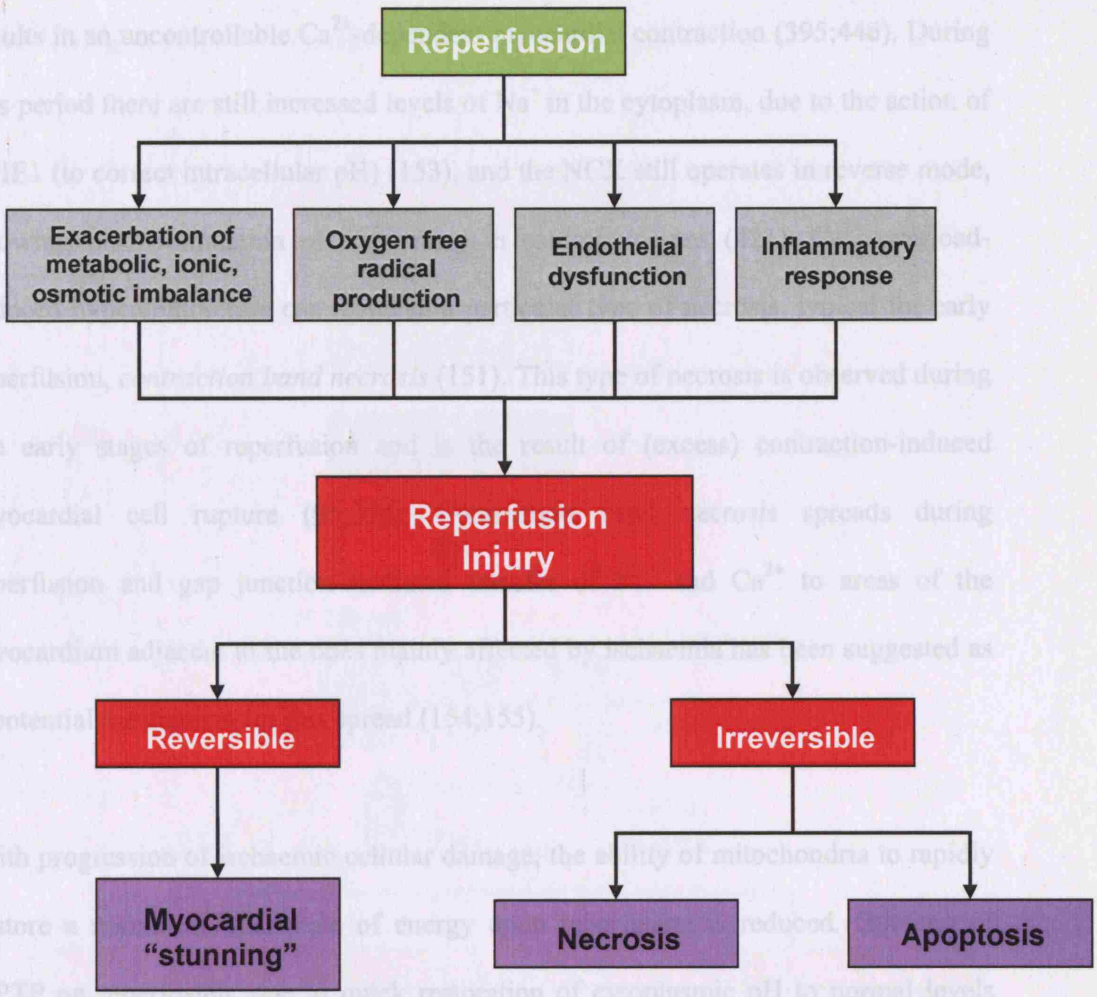


Figure 1.5 Mechanisms and mediators of reperfusion injury. Adapted from Verma *et al*, Circulation; 105: 2332, 2002.

1.2.2.2.1 Role of disruption of metabolic, ionic and osmotic balance in reperfusion injury

At the end of ischaemia, cardiomyocytes are in a state of cytoplasmic Ca^{2+} overload *(section 1.2.2.1.2)*. Reoxygenation brought on by reperfusion leads to a rapid recovery of oxidative energy production. Re-synthesis of ATP enables cardiomyocytes to recover from the loss of cytosolic cation balance but it also reactivates the contractile

machinery. Contractile activation is normally faster than Ca^{2+} recovery, and this results in an uncontrollable Ca^{2+}-dependent myocardial contraction (395;446). During this period there are still increased levels of Na^{+} in the cytoplasm, due to the action of NHE1 (to correct intracellular pH) (153), and the NCX still operates in reverse mode, allowing the continuation of Ca^{2+} entry in cardiomyocytes (421). Ca^{2+} overload-induced hypercontracture can result in a particular type of necrosis, typical for early reperfusion, *contraction band necrosis* (151). This type of necrosis is observed during the early stages of reperfusion and is the result of (excess) contraction-induced myocardial cell rupture (95;394). Contraction band necrosis spreads during reperfusion and gap junction-mediated transfer of Na^{+} and Ca^{2+} to areas of the myocardium adjacent to the ones mainly affected by ischaemia has been suggested as a potential mechanism for this spread (154;155).

With progression of ischaemic cellular damage, the ability of mitochondria to rapidly restore a normal cellular state of energy upon reperfusion is reduced. Opening of mPTP on reperfusion, due to quick restoration of cytoplasmic pH to normal levels (intracellular acidosis inhibits mPTP opening in ischaemia) and the production of ROS *(see section 1.2.2.2.2)*, depolarises mitochondria (disruption of energy production) and causes mitochondrial swelling that leads to the rupture of the outer mitochondrial membrane *(figure 1.6)* (184). Recent evidence suggests that the extent of mPTP opening during reperfusion may determine whether the injury suffered by cardiomyocytes is reversible ("stunned" myocardium) or irreversible with associated myocardial cell death (98;196;514). Limited opening of mPTP upon reperfusion (following less severe ischaemia) may result in temporary disruption of energy production which is restored with time. In contrast, generalised and prolonged

opening of mPTP during reperfusion (following severe ischaemia) results in mitochondrial rupture and ultimate myocardial necrosis *(figure 1.6)*.

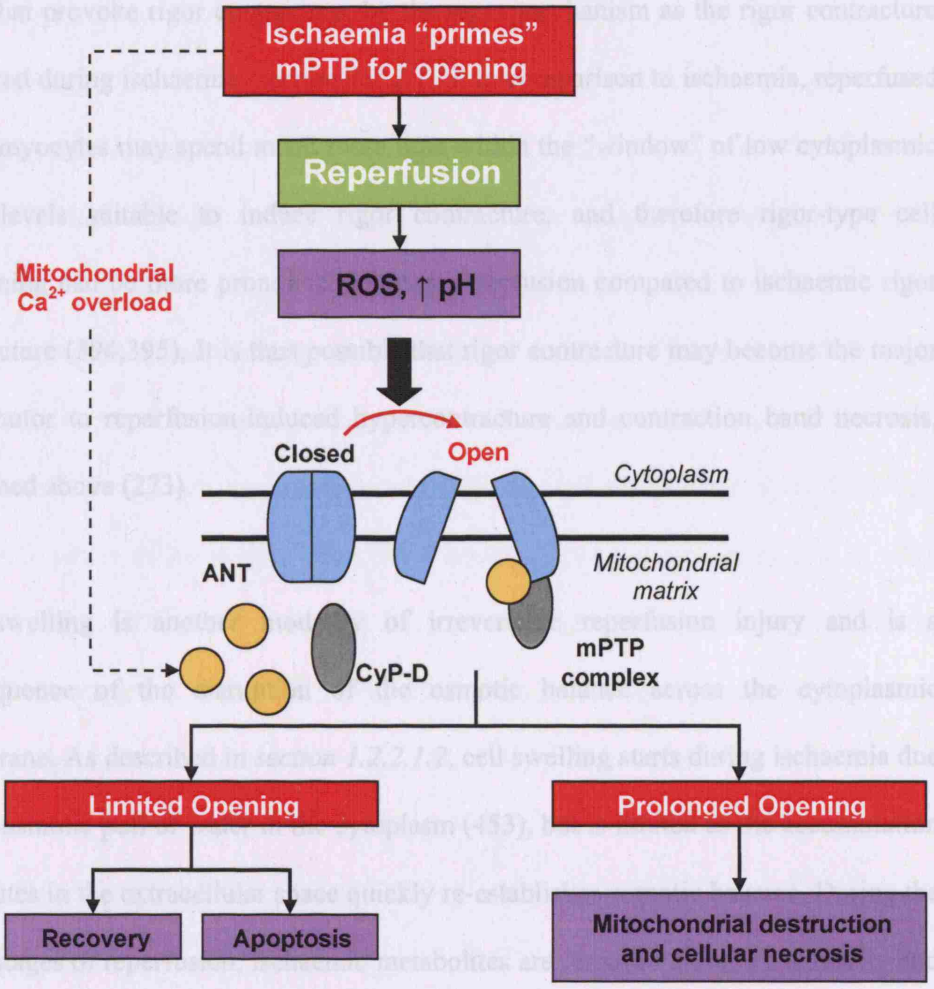


Figure 1.6 Role of mitochondrial permeability transition pore *(mPTP)* in reperfusion injury. Adapted from Weiss *et al*, Circ Res; 393: 292, 2003. *ROS: reactive oxygen species; CyP-D: cyclophilin-D.*

Even when the myocardium has retained the ability to produce ATP, energy production is may be limited during the early stages of reperfusion. As a result, during early reperfusion cardiomyocytes contain very low (although rising) concentrations of ATP that provoke rigor contracture, by the same mechanism as the rigor contracture observed during ischaemia *(section 1.2.2.1.1)*. In comparison to ischaemia, reperfused cardiomyocytes may spend much more time within the "window" of low cytoplasmic ATP levels suitable to induce rigor contracture, and therefore rigor-type cell shortening can be more pronounced during reperfusion compared to ischaemic rigor contracture (394;395). It is thus possible that rigor contracture may become the major contributor to reperfusion-induced hypercontracture and contraction band necrosis, described above (273).

Cell swelling is another modality of irreversible reperfusion injury and is a consequence of the disruption of the osmotic balance across the cytoplasmic membrane. As described in *section 1.2.2.1.2*, cell swelling starts during ischaemia due to the osmotic pull of water in the cytoplasm (453), but is limited as the accumulation of solutes in the extracellular space quickly re-establishes osmotic balance. During the early stages of reperfusion, ischaemic metabolites are removed and the osmolarity and pH of the extracellular fluid returns to normal levels. In the cytoplasm, despite reperfusion, there are still high concentrations of Na^+ (via NHE1 activity during reperfusion) (11), Ca^{2+} (via high cytoplasmic Na^+-induced reverse action of NCX) (421) and metabolites produced during ischaemia and this osmotic in-balance between the intra- and extracellular space results in excess water entering the cardiomyocytes. The latter together with the increased cellular fragility, due to enzymatic or ROS-

mediated structural damage, results in reperfusion-induced catastrophic swelling and subsequent myocardial cell necrosis (116;223;227).

1.2.2.2.2 Role of reactive oxygen species in reperfusion injury

One of the hallmarks of reperfusion injury is the generation of reactive oxygen species (ROS) upon reoxygenation (55;292). Evidence supporting a role for ROS in reperfusion-induced injury is indirect, and based on the reduction in reperfusion injury caused by antioxidants such as DMSO (152), superoxide dismutase and catalase (232). However, other investigators have failed to observe such results, possibly attributed to several factors including species and experimental technique differences, as well as differences in the type/properties and dose of antioxidants used (135;408). The early phase of blood flow restoration seems to play an important role in ROS production and ROS are produced in the greatest amount during the first minutes of reperfusion *(figure 1.7)* (15;495;551), although their over-production may continue for longer periods.

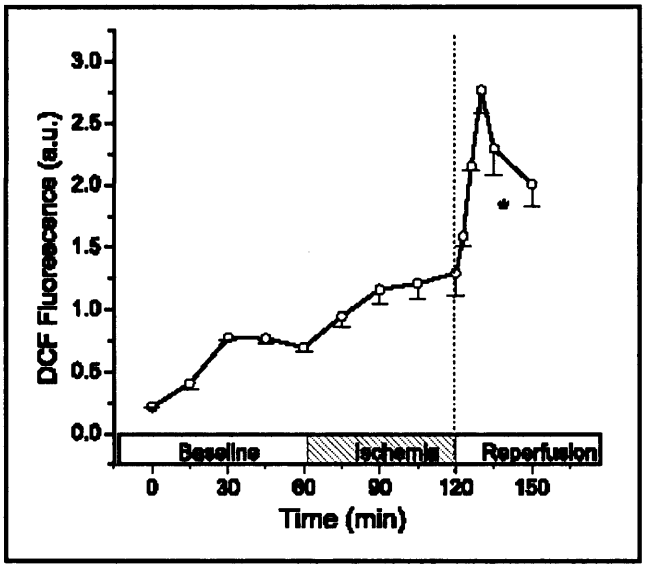


Figure 1.7 Original data demonstrating the "burst" of ROS during reperfusion in cardiomyocytes. ROS production peaks during the first minutes of reperfusion. ROS measurement was performed using the fluorescent oxidant probe 2,7-dichlorofluorescin (DCF) (Vanden Hoek *et al*, J Mol Cell Cardiol; 29: 2571, 1997).

ROS include free radicals [one unpaired electron that makes them chemically reactive; e.g. superoxide anion (O_2^-), hydroxyl radical (·OH)], and hydrogen peroxide (H_2O_2) (128). O_2^-· production usually involves a one-electron reduction of molecular O_2. The negatively charged O_2^-· radical is unstable in aqueous solution (half-life of a few seconds) and is rapidly dismutated to H_2O_2. It can undergo several chemical reactions depending on the concentration, localization and proximity to other radicals and enzymes. O_2^-· reacts rather poorly with itself to produce H_2O_2 and O_2 (rate constant 8×10^4 mol·l^{-1}·s^{-1}), but this reaction is substantially accelerated (rate constant 2×10^9 mol·l^{-1}·s^{-1}) by superoxide dismutase (cytoplasmic, Cu,ZnSOD; mitochondrial MnSOD) (300). Thus, at low O_2^-· concentrations (picomolar range), most biological effects are likely to be secondary to H_2O_2 production. Indeed, H_2O_2 is more stable and

diffusible than $O_2^{-}\cdot$ and is more cell membrane permeable and may therefore be more biologically relevant than $O_2^{-}\cdot$. $O_2^{-}\cdot$ reacts with nitric oxide (NO) at a significantly faster rate than with SOD (rate constant 7×10^9 $mol \cdot l^{-1} \cdot s^{-1}$), so that when levels of NO are in the high nanomolar range, NO may out-compete SOD and react with $O_2^{-}\cdot$ to generate peroxynitrite ($ONOO^{-}\cdot$) (452). When $O_2^{-}\cdot$ levels are higher still, it can react with iron-sulphur centres in many proteins to release ferrous iron (Fe^{2+}) which, together with Fe^{2+} released during ischaemia by degradation of mitochondrial complexes I and II *(section 1.2.2.1.3)*, reacts with H_2O_2 to produce the highly reactive ·OH radical (Fenton reaction) (34;75). Chelation of iron by deferoxamine during reperfusion has been shown to reduce injury in animal models of myocardial IR (57;309). Sources of ROS in the myocardium during reperfusion include xanthine oxidase (XO) (68), the mitochondrial electron transport system (14), uncoupled nitric oxide synthase (NOS) (300), and NADPH oxidase (207).

Xanthine oxido-reductase (XOR) is a ubiquitous metalloflavoprotein found as one of two inter-convertible yet functionally distinct forms, namely xanthine dehydrogenase (XD), which is constitutively expressed in vivo, and xanthine oxidase (XO), which is generated by the post-translational modification of XD (45). Functionally, both XD and XO catalyse oxidation of hypoxanthine to xanthine and xanthine to urate (373). However, whereas XD requires NAD^+ as an electron acceptor, XO instead requires the reduction of molecular O_2, thereby generating $O_2^{-}\cdot$ (191;203). The conversion of XD to XO occurs via irreversible proteolytic cleavage of a segment of XD during ischaemia. The latter in association with the high intracellular levels of hypoxanthine and xanthine (generated by adenine nucleotide degradation during ischaemia) and the re-introduction of oxygen, favour the production of $O_2^{-}\cdot$ by XO during reperfusion

(45). XO has been identified in the bovine (108) and rat heart (349), and some (78;349), but not all (307), studies have identified XO protein and/or activity in the human heart. In addition, XO has been uniformly identified in animal and human endothelial cells (333) and circulating XO has been demonstrated in human, rat and rabbit serum (45). The role of XO in ROS production during reperfusion is supported by studies demonstrating increased activity of the enzyme during reperfusion in animal models of myocardial infarction (68;143). Endothelial XO activity is also increased during reperfusion (300) and in combination with IR-induced elevation in circulating XO (359) may contribute to ROS-mediated myocardial injury. The XO inhibitor allopurinol attenuated injury when administered during reperfusion in animal models of myocardial IR (51;517) and reduced reperfusion injury in the human myocardium in the setting of open cardiac surgery (163) and primary coronary angioplasty (179).

The mitochondria are another source of ROS (34;221). Ischaemic disruption to components of the electron transport chain, results in electron leakage, which continues during reperfusion and favours the formation of $O_2^{-}\cdot$ *(section 1.2.2.1.3).* Production of $O_2^{-}\cdot$ mainly occurs at complexes I (NADH coenzyme Q reductase) and III (ubiquinol cytochrome c reductase) (458;488;489). ROS production in cardiomyocytes is reduced during reperfusion by administration of metabolic inhibitors of electron transport at complex I (amytal or rotenone) or complex III (myxothiazol) (34). However, not all investigators are in agreement about the role of mitochondrial ROS production during reperfusion. It has been reported that ROS are also generated during ischaemia, and mitochondrial electron transport complexes have implicated as the main source (35).

ROS are also generated during reperfusion by nitric oxide synthase (NOS). NOS is a complex homodimeric oxido-reductase that generates $O_2^{-}\cdot$ rather than NO under some conditions (33;427). The endothelial subtype of NOS (eNOS, NOS3) has been found in cardiomyocytes (62;325;369) and may be involved in myocardial ROS production via an uncoupling mechanism. The essential eNOS cofactor tetrahydrobiopterin (BH_4) appears to have a key role in regulating NOS function by "coupling" the reduction of molecular O_2 to L-arginine oxidation as well as maintaining the stability of NOS dimers (498). Thus BH_4 availability may be a crucial factor in the balance between NO and $O_2^{-}\cdot$ generation by NOS (278). BH_4 itself is highly susceptible to oxidative degradation, and the initial oxidative loss of BH_4 in response to increased ROS production from other sources during reperfusion may be responsible for the amplification of reperfusion-induced oxidative injury through increased NOS-dependent $O_2^{-}\cdot$ generation (282). NOS-derived NO may react with superoxide anions to produce the potent oxidant $ONOO\cdot^{-}$, which is also implicated in ROS-induced injury during reperfusion (506;550). Decreased bioavailability of NO, either due to decreased synthesis or due to scavenging of ROS, can complicate IR injury via pathophysiological mechanisms involving blood vessels (the vascular endothelium is the main site of NO production), and will be discussed in *section 1.2.2.2.3.*

NADPH oxidase may also generate ROS during reperfusion. NADPH oxidase is an $O_2^{-}\cdot$ producing enzyme that is central to the microbial killing mechanisms of phagocytic leukocytes (neutrophils) (433). The prototypic NADPH oxidase consists of a membrane associated cytochrome b_{558}, composed of one $gp91^{phox}$ and a $p22^{phox}$ subunit and several cytosolic regulatory subunits ($p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and the small G-protein Rac1) that translocate to the membrane and associate with

cytochrome b_{558} upon neutrophil activation (23). The latter process rapidly activates the oxidase, utilising NADPH to produce $O_2^{-}\cdot$. Neutrophils have been involved in the pathophysiology of IR injury *(see section 1.2.2.2.4)*, and ROS produced by NADPH oxidase, following the infiltration of neutrophils in previously ischaemic myocardium during reperfusion, may be responsible for oxidative injury in later stages of reperfusion (501). However, NADPH oxidase has also been identified in non-phagocytic tissues, including the myocardium, and the vascular wall (171). All the classical neutrophil NADPH subunits are expressed in cardiomyocytes (199) and endothelial cells (166;233;299) but recently several isoforms of $gp91^{phox}$, termed Nox (for non-phagocytic oxidase), have been identified (275;280). To date, the Nox family comprises of five members (Nox 1-5) from which Nox 2 is $gp91^{phox}$ (neutrophil isoform) (300). In addition to Nox 2, Nox 4 has been identified in cardiomyocytes (170) and endothelial cells (9). Although the role of non-phagocytic NADPH oxidase in myocardial IR injury is not clear at present (206), it may contribute to the production of ROS in cardiomyocytes during early reperfusion and thus contribute to oxidative injury (207;216;549).

ROS exert many deleterious effects on myocardial tissue. They react with proteins, membrane lipids, and nucleic acids (239). Proteins are attacked primarily by ·OH, and oxidation of proteins changes their structure and function. Membrane ionic channels change their permeability to Ca^{2+}, K^{+} and to a lesser extent to Na^{+}. Reverse action of the NCX has been reported to be stimulated in the presence of ROS (133). Activity of ion pumps like the Na^{+}/K^{+} pump (528) or pumps involved in the transport of Ca^{2+} to the sarcoplasmic reticulum is decreased following exposure to oxidative stress (527). The above could be responsible for the prolonged ionic and metabolic in-balance

observed during the early stages of reperfusion. In addition, oxidative modification of contractile proteins may cause contractile dysfunction and haemodynamic failure (397;462). ROS generated during reperfusion can induce peroxidation of membrane lipids in cardiomyocytes (12;371), and result in membrane failure. Destruction of nucleic acid by ROS has also been reported to occur during reperfusion. Mitochondrial DNA is particularly sensitive to such injury as it is in direct proximity to one of the major sites of ROS production (239). Another effect of ROS on the mitochondria is the promotion of the opening of the mPTP that can result in cardiomyocyte death as described in *section 1.2.2.2.1* (184).

Cardiomyocyte anti-oxidant defences are compromised by ischaemia and subsequent reperfusion; under normal physiological conditions, ROS are scavenged by endogenous antioxidants, including SOD, catalase and glutathionine peroxidase (114). Ischaemia damages antioxidant enzymes and natural anti-oxidant defences may be overwhelmed by the burst of ROS during the early stages of reperfusion (114;189).

1.2.2.2.3 Role of the vascular endothelium in reperfusion injury

The consequences of ischaemia and reperfusion injury also extend to the vascular wall and especially the endothelium (79). This lines the inner surface of blood vessels, forming a dynamic structure that is essential for both vascular and myocardial homeostasis. Ischaemia depletes energy stores in endothelial cells and disrupts endothelial cell ionic/osmotic balance and cytoskeletal organisation, via mechanisms similar to those in cardiomyocytes (79;234;431). These changes are accompanied by diminished production of mediators such as nitric oxide and prostaglandins, and accelerated production of others (e.g. endothelin, thromboxane A2) (79). Many of

these endothelial cell responses to ischaemia are exacerbated by reperfusion and lead to reversible or irreversible endothelial cell injury and disruption of the endothelial cell barrier (271;362).

In isolated coronary (conduit) arteries, ischaemia and reperfusion is accompanied by decreased endothelium-dependent relaxation, while the response to endothelium independent relaxants remains intact (263;379;494). Similar observations have been made in coronary resistance vessels (arterioles) (28;192). Ischaemic episodes that are not severe enough to result in endothelial damage have been shown to be followed by endothelial dysfunction on reperfusion (407). This suggests that, under certain circumstances, endothelial dysfunction is a manifestation of reperfusion injury (502). The decreased bioavailability of nitric oxide, due to decreased endogenous production or increased inactivation by free radicals produced during reperfusion by enzymes, such as XO and NADPH oxidase, or the mitochondria *(see section 1.2.2.2.2)*, contributes to the development of endothelial dysfunction associated with reperfusion (79;178;192). Constitutive nitric oxide production continuously opposes vasoconstrictor influences in conduit and resistance vessels and, in concert with other endothelium derived factors, acts as an inhibitor of platelet aggregation (79;427). Consequently, endothelial dysfunction, following myocardial ischaemia and reperfusion, may lead to increased vasoconstriction and platelet aggregation in coronary arteries and/or cardiac arterioles, and thus prevent restoration of normal blood flow to previously ischaemic cardiac myocytes (via vasospasm or thrombosis respectively) *(figure 1.8)*.

Endothelial dysfunction has also been associated with the development of the acute inflammatory response observed during reperfusion. This includes increased vascular permeability, accumulation of interstitial fluid (oedema), compression of the microvasculature and 'plugged capillaries' *(see section 1.2.2.2.4)*, resulting in capillary malperfusion (termed the "no-reflow" phenomenon) (406), which can further impair restoration of normal blood flow to previously ischaemic myocardium *(figure 1.8)*. In addition to other functions, NO is an inhibitor of neutrophil activation and adhesion (264) and decreased NO bioavailabity during reperfusion triggers the adhesion of neutrophils to endothelial cells through the induction of adhesion molecules *(see section 1.2.2.2.4)*. The subsequent infiltration of neutrophils in the peri-vascular tissue results in the release of ROS, proteases and elastases, leading to cell damage (148).

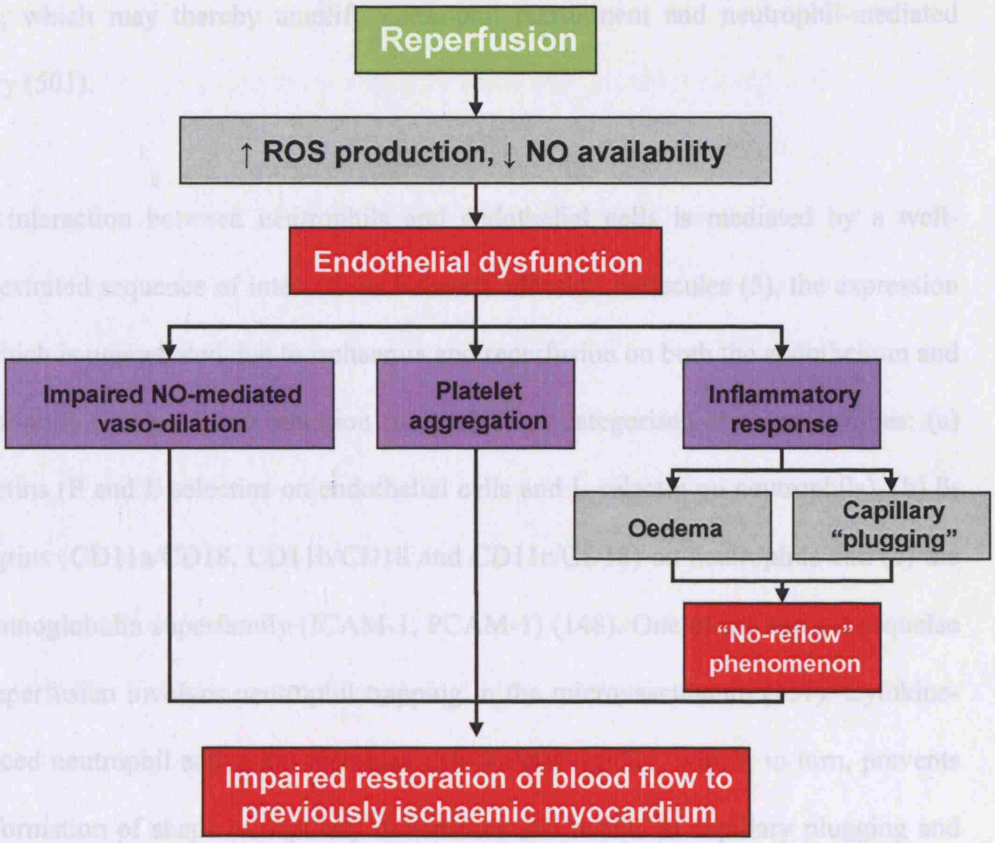


Figure 1.8 Effect of reperfusion induced endothelial dysfunction on blood flow restoration to previously ischaemic myocardium.

1.2.2.2.4 Role of neutrophils in reperfusion injury

Neutrophils play a central role in the inflammatory response to reperfusion. Ischaemia and reperfusion activate complement (C5a) (19;127), release histamine, and pro-inflammatory cytokines (TNF-a, IL-6, IL-8) that activate neutrophils and recruit them to sites of injury (147). Activated neutrophils release oxidants (superoxide, hydrogen peroxide, hypochlorous acid) and proteases (elastase, collagenase) that further damage cells. They also release inflammatory products (LTB_4, PAF, TXA_2 TXB_2) that are potent stimulants of neutrophil chemotaxis, degranulation and adhesion to endothelial

cells, which may thereby amplify neutrophil recruitment and neutrophil-mediated injury (501).

The interaction between neutrophils and endothelial cells is mediated by a well-orchestrated sequence of interactions between adhesion molecules (5), the expression of which is upregulated due to ischaemia and reperfusion on both the endothelium and neutrophils (214). These adhesion molecules are categorised in three families: (a) selectins (P and E selectins on endothelial cells and L selectin on neutrophils), (b) β_2 integrins (CD11a/CD18, CD11b/CD18 and CD11c/CD18) on neutrophils and (c) the immunoglobulin superfamily (ICAM-1, PCAM-1) (148). One of the earliest sequelae of reperfusion involves neutrophil trapping in the microvasculature (137). Cytokine-induced neutrophil activation increases cytoskeletal rigidity, which, in turn, prevents conformation of shape to capillary dimensions and results in capillary plugging and the "no-reflow" phenomenon *(see section 1.2.2.2.3)* (406). Neutrophils interact with the endothelium through a process of "rolling" which is mediated by endothelial P-selectin (291) and L-selectin and/or sialylated glycoprotein on the neutrophil (PSGL-1) *(figure 1.9)* (329;505). This initial loose adherence is an obligatory step that is necessary for later firm adherence, mediated by ICAM-1 (constitutively expressed on endothelial cells), E-selectin (expressed on endothelial cells after 4-6 hours of reperfusion) and β_2 integrins (148). This leads to transendothelial migration (diapedesis) of neutrophils into the tissue parenchyma, mediated by endothelial PCAM-1 *(figure 1.9)* (501). The release of reactive oxygen species from NADPH oxidase and proteolytic enzymes, that follows, leads to tissue injury and necrosis.

The pathogenic role of neutrophils in ischaemia reperfusion injury is indicated by the reduction in IR injury due to reduction in neutrophil numbers (using anti-neutrophil antibodies (411), neutrophil depleting anti-metabolites (343), or neutrophil filters (136)). In addition, direct interference with the early interactions between neutrophils and endothelium have also been effective in reducing post-ischaemic injury. Antibodies against P- and L-selectins (319;518), CD11/CD18 complex (318;448) and ICAM-1 (317;533) have all been reported to attenuate neutrophil accumulation and adhesion, resulting in reduction of endothelial dysfunction and infarct size in experimental models of myocardial ischaemia and reperfusion. These observations further substantiate the role of neutrophils in mediating the distal events of infarction and blood flow defects that are characteristic of severe post ischaemic injury, although not all studies to date support this (29).

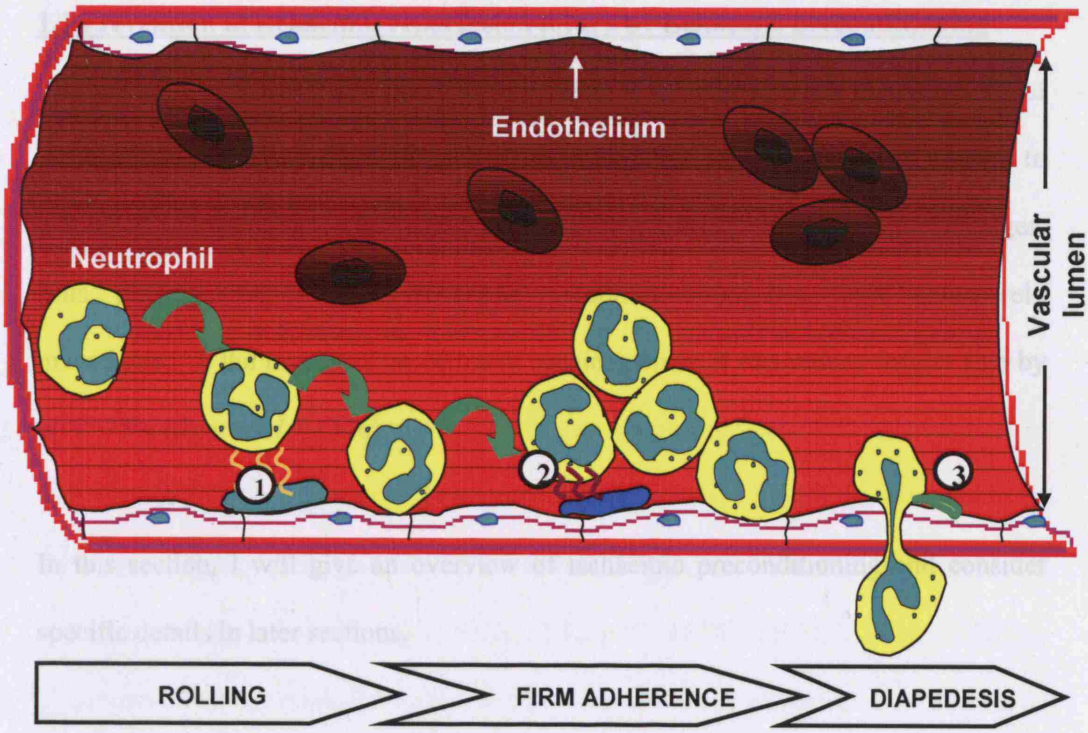


Figure 1.9 Neutrophil-endothelial interactions during reperfusion. Neutrophils interact with the endothelium through a process of "rolling" which is mediated by endothelial P-selectin and neutrophil L-selectin *(1)*. Firm adherence of neutrophils follows and is mediated by endothelial E-selectin and ICAM-1, and neutrophil β_2-integrins *(2)*. The final step is diapedesis of neutrophils into tissue parenchyma, mediated by PCAM-1 within the endothelial cell junctions *(3)*. Adapted from Collard and Gelman, Anesthesiology; 94:1133, 2001.

1.3 Prevention of ischaemia-reperfusion injury by ischaemic preconditioning

The experimental work described in this thesis is concerned with the reduction of ischaemia-reperfusion injury by ischaemic preconditioning. Exposure of tissues to sub-lethal ischaemia protects against damage during subsequent ischaemic challenges. This phenomenon, termed ischaemic preconditioning, has been extensively investigated in the myocardium of many species, where it can reduce infarct size by up to 75% (*figure 1.10)* (346).

In this section, I will give an overview of ischaemic preconditioning and consider specific details in later sections.

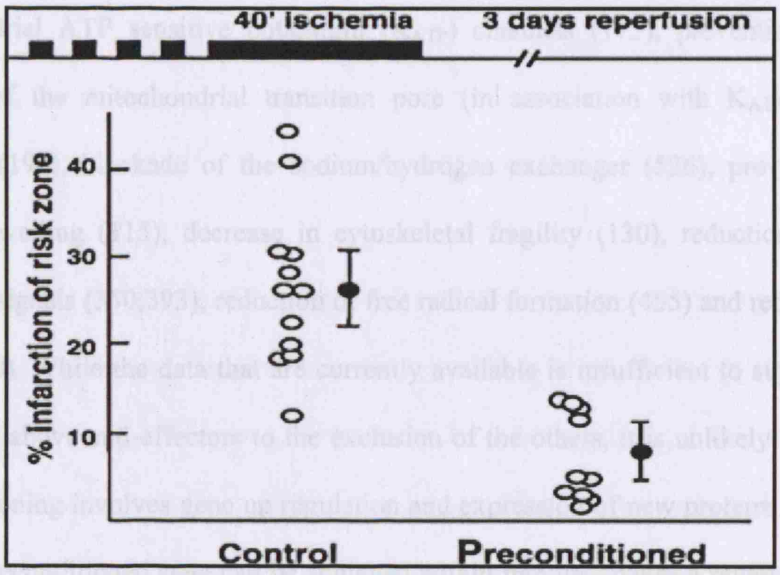


Figure 1.10 Original data demonstrating protection by ischaemic preconditioning against myocardial IR injury (Murry *et al*, Circulation; 74: 1124, 1986).

An early phase of ischaemic preconditioning ("classic" preconditioning) occurs within minutes of the preconditioning stimulus. The preconditioned state is very transient and lasts for only 1-2 hours in anaesthetised animals (347;417) and is lost somewhere between 2-4 hours in some species *(figure 1.12)* (72). The early phase of preconditioning is triggered by a number of stimuli that are generated during hypoxia, including adenosine, bradykinin, endogenous opiates and reactive oxygen species (538). These mediators act on G_i coupled cell surface receptors to initiate a cascade of second messengers, including activation of phospholipases C and D, stimulation of the ε isoform of protein kinase C (PKCε) (308;391), and activation of tyrosine and mitogen activated protein kinases (ERK, JNK, p38 MAPK) (390). The end-effectors of preconditioning remain uncertain and include metabolic effects (348), opening of mitochondrial ATP sensitive potassium (K_{ATP}) channels (173), prevention of the opening of the mitochondrial transition pore (in association with K_{ATP} channel opening) (194), blockade of the sodium/hydrogen exchanger (526), prevention of osmotic swelling (115), decrease in cytoskeletal fragility (130), reduction of pro-apoptotic signals (350;393), reduction of free radical formation (455) and reduction of TNF-a (40). While the data that are currently available is insufficient to support any one of the above end-effectors to the exclusion of the others, it is unlikely that early preconditioning involves gene up regulation and expression of new proteins. The fact that the preconditioned state can be achieved within minutes makes a reversible post-translational modification (phosphorylation or translocation) of some pre-existing protein more likely. The mechanisms of early ischaemic preconditioning are summarised in *figure 1.11*. Certain of these aspects of preconditioning are discussed in more detail in *section 1.4.3*.

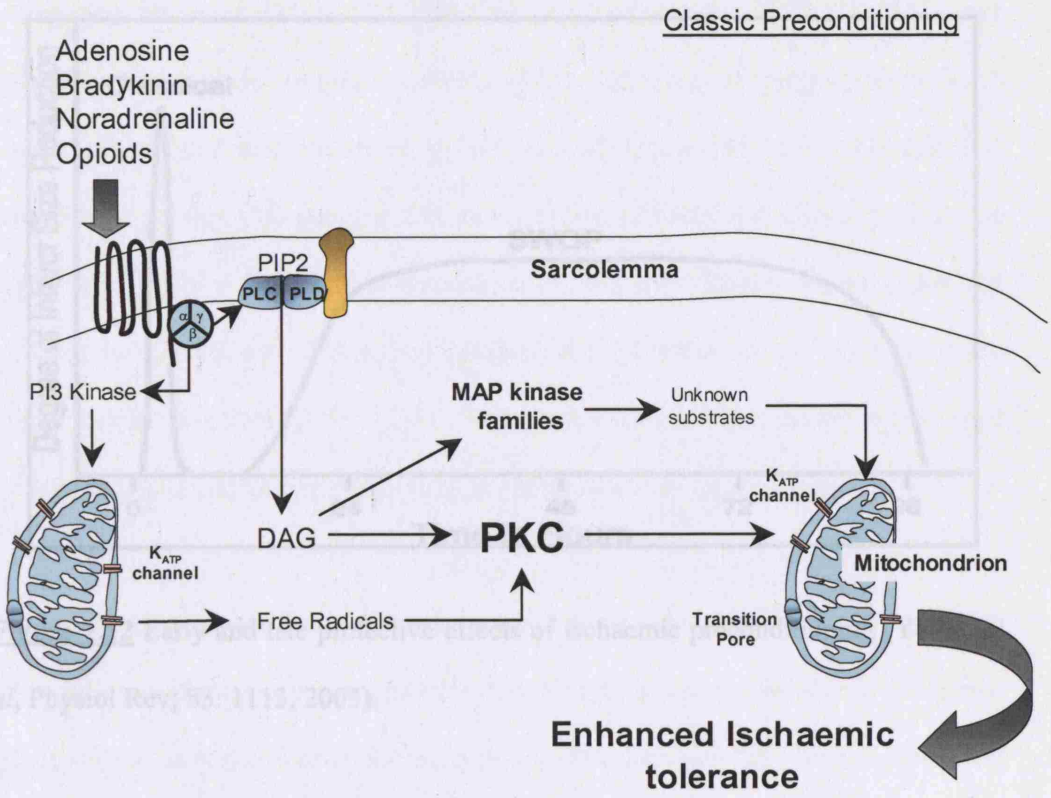


Figure 1.11 Mechanisms of early ("classic") ischaemic preconditioning.

A late phase of preconditioning occurs 24 hours after the preconditioning stimulus, which is more prolonged than the early phase and lasts up to 72 hours *(figure 1.12)* (31;270;322). This delayed phase of resistance to ischaemic injury has been termed second window of protection (SWOP), distinguishing it from early or "classic preconditioning" (322). The prolonged (24-hour) interval between the preconditioning event and its renewed protection one day later allows for the possibility of new protein synthesis, post-translational protein modification, and a change in the compartmentalisation of existing proteins (537).

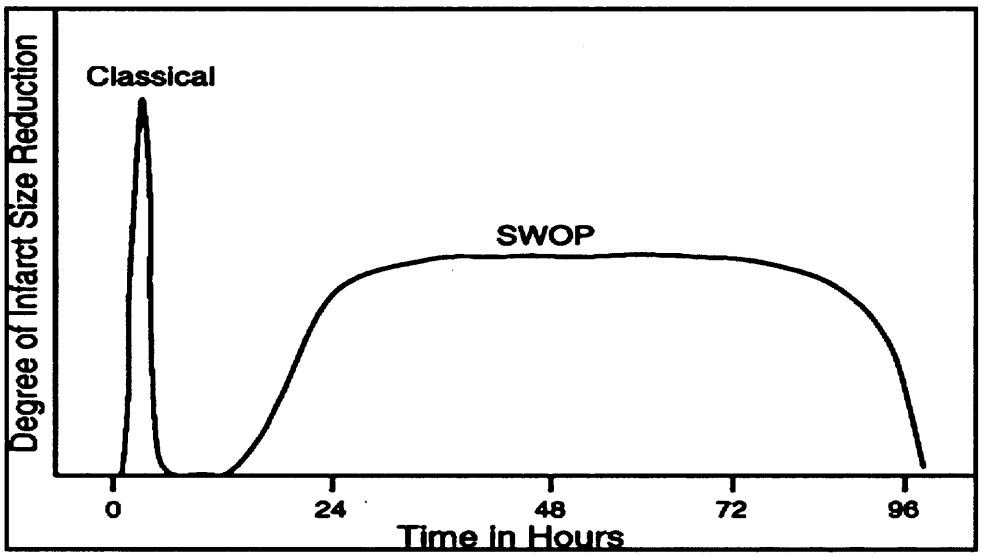


Figure 1.12 Early and late protective effects of ischaemic preconditioning (Yellon *et al*, Physiol Rev; 83: 1113, 2003).

A wide range of stimuli can induce the second window of preconditioning. These are somewhat similar to the triggers of the early preconditioning and include adenosine, bradykinin, endogenous opiates, nitric oxide, cytokines and reactive oxygen species (538). They are produced in response to transient ischaemia and reperfusion and activate multiple signal cascades. The ε isoform of protein kinase C, as in "classic" preconditioning, is also an essential mediator of the second window of protection (401). Other mediators, with possible downstream positions in relation to PKCε, are tyrosine kinases Src and Lck (392) and mitogen activated protein kinases (ERK, JNK, p38 MAPK) (25). The transcriptional regulator NF-κB may well be the common pathway through which the multiple signals generated by ischaemic preconditioning initiate cardiac and other tissue gene expression (538). Inhibition of NF-κB abolished the second window of preconditioning (531). Second window protection is associated with increased expression of heat shock proteins (hsp 27, 70 and 72i) (105;255;539),

antioxidant enzymes (MnSOD) (209;270), cyclooxygenase (COX-2) (442) and inducible nitric oxide synthase (iNOS) (465). Opening of mitochondrial K_{ATP} channels has also been implicated in late preconditioning (44;466). Although it is unproven at present, it is possible that up regulation of protein(s) associated with the K_{ATP} channel alters its opening characteristics, and this determines the protection afforded by delayed preconditioning (possibly by inhibition of the opening of the mitochondrial transition pore) (538). The mechanisms of the second window of protection by ischaemic preconditioning are summarised in *figure 1.13.*

In the vascular endothelium, although research has not been as extensive as for the myocardium, ischaemic preconditioning has also been shown to have a protective effect against ischaemia and reperfusion injury (281). Investigators have mainly used rat models of myocardial infarction, and have shown that ischaemic preconditioning can protect against endothelial dysfunction and endothelial structural damage when short periods of ischaemia and reperfusion are applied immediately before or 24 hours before index ischaemia (235;407), thus demonstrating both early and late phases of ischaemic preconditioning. The triggers, mediators and end effectors have been shown to be the same as for ischaemic preconditioning in the myocardium (415).

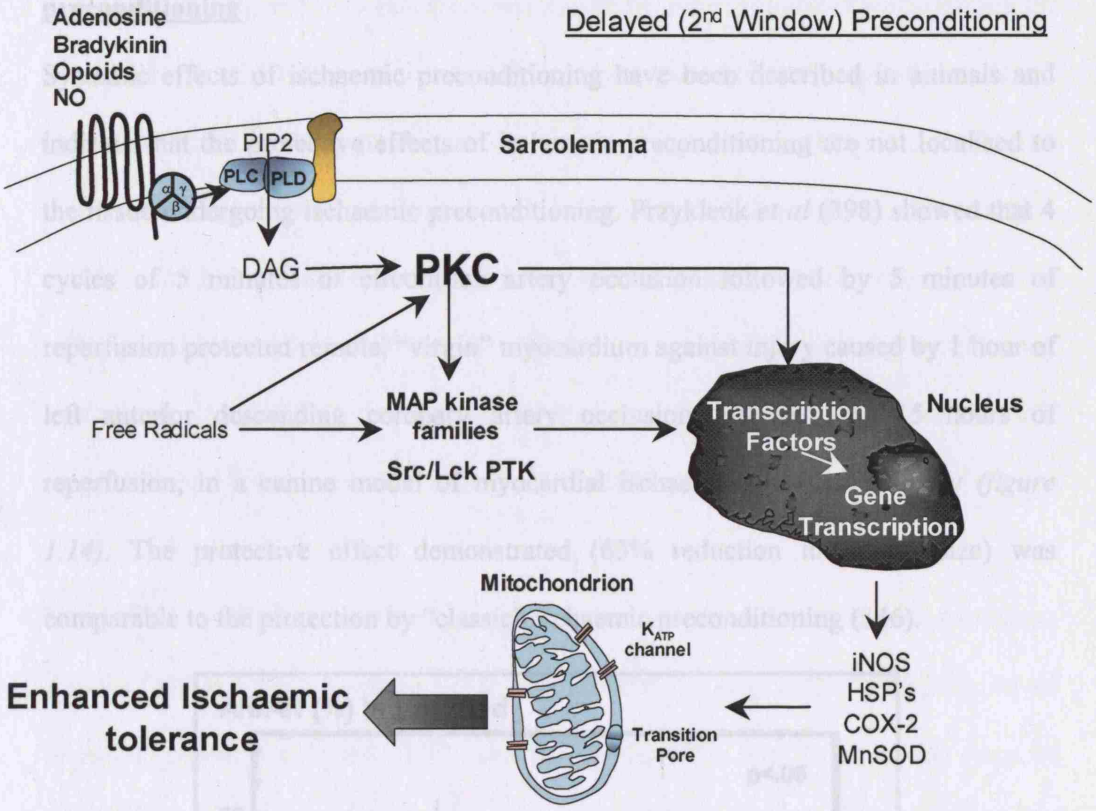


Figure 1.13 Mechanisms of late ("second-window") protection by ischaemic preconditioning.

1.4 Prevention of ischaemia-reperfusion injury by *remote* ischaemic preconditioning

Systemic effects of ischaemic preconditioning have been described in animals and indicate that the protective effects of ischaemic preconditioning are not localised to the tissue undergoing ischaemic preconditioning. Przyklenk *et al* (398) showed that 4 cycles of 5 minutes of circumflex artery occlusion followed by 5 minutes of reperfusion protected remote, "virgin" myocardium against injury caused by 1 hour of left anterior descending coronary artery occlusion followed by 4.5 hours of reperfusion, in a canine model of myocardial ischaemia-reperfusion injury *(figure 1.14)*. The protective effect demonstrated (63% reduction in infarct size) was comparable to the protection by "classic" ischaemic preconditioning (346).

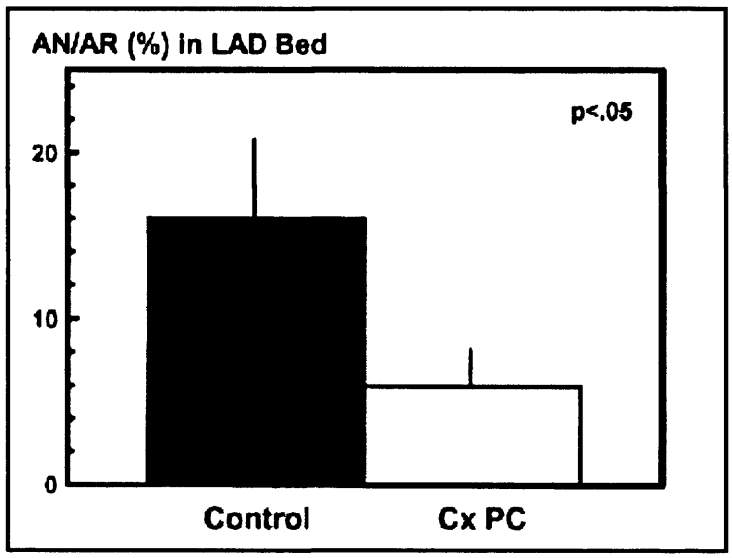


Figure 1.14 Original data demonstrating protection of "virgin" myocardium, against IR injury, by ischaemic preconditioning on a remote (at a distance) part of the heart. (Przyklenk *et al*, Circulation; 87: 893, 1993). *AN: area of necrosis; PC: preconditioning; LAD: left anterior descending coronary artery; Cx: circumflex coronary artery.*

Since then RIPC has been described in rat (377), mouse (478), pig (243), rabbit (467), dog (303), and sheep (524) models of experimental ischaemia and reperfusion injury. The majority of these studies have demonstrated the protective effects of RIPC against myocardial ischaemia-reperfusion injury both in vivo and in vitro (399). The main end point used was extent of myocardial tissue necrosis (as assessed by staining viable tissue with tetrazolium staining or by measuring known markers of myocardial injury in the blood), but functional (presence of reperfusion tachy-arrhythmias, myocardial contractility), and metabolic (rate of ATP depletion, intracellular pH, intracellular Ca^{2+} concentration) end points have also been used (313;399).

In addition, RIPC has been shown to be protective against ischaemia-reperfusion injury of the kidney (rat, mouse) (20;372), muscle (rat, pig, dog) (7;268;303), small intestine (rabbit – in vitro) (120), and brain (rat) (37). Similar endpoints (size of necrotic area, markers of functional recovery, markers of inflammatory response), adapted for the organ studied in each particular case, were used. The majority of studies have clearly established the presence of systemic protective effects by ischaemic preconditioning (399). A systematic review of the literature on remote ischaemic preconditioning is summarised in *Appendix A.*

1.4.1 Remote ischaemic preconditioning stimulus

As for local ischaemic preconditioning, remote ischaemic preconditioning requires a brief period of ischaemia followed by reperfusion to trigger the response in rabbits and rats (161;328). The remote ischaemic preconditioning stimulus has been applied in a variety of tissues and/or organs that are distant to ones that undergo ischaemia reperfusion injury *(see tables A.1 to A.8, Appendix A).* The majority of studies to date

have used short periods of intestinal ischaemia to induce RIPC of the myocardium in rat (520) and rabbit (471) models. A variety of occlusion regimes were applied in these studies that include 15 minutes of mesenteric artery (MA) occlusion followed by 10 minutes of reperfusion (423) or 3 cycles of 5 minutes of mesenteric ischaemia and reperfusion (378). Similar results have been obtained using preconditioning ischaemia and reperfusion in the liver in rats (69), and the kidney in rabbit and rat models (449;467). 10 and 15 minutes of renal (161;381) or hepatic (20) artery occlusion were shown to reduce ischaemia-reperfusion induced necrosis in the myocardium (381;467) and the kidney (20). Studies using renal ischaemia and reperfusion models have provided conflicting results (372), but these could be attributed to model differences (rat vs. mouse) , or even inappropriate "timing" between the RIPC stimulus and the prolonged period of ischaemia *(see section 1.4.2)*. One study that applied the RIPC stimulus to the brain failed to demonstrate a systemic protective effect (109), although it should be noted that in another study unilateral middle cerebral artery occlusion could induce RIPC-like protection on the contralateral cerebral hemisphere (37).

There may be a dose-response relationship between the ischaemic stimulus and degree of protection of RIPC. In an in vivo model of myocardial infarction, 5, 10 or 15 minutes of infra-renal occlusion of the aorta reduced myocardial injury, with greater protection from the fifteen-minute occlusion compared to the shorter periods of preconditioning ischaemia (512). In general, a threshold stimulus producing "all-or-none" protection has been reported most often for local ischaemic preconditioning, with "stronger" stimulus unable to cause additional protection (220;298;429). At present it is difficult to be certain if RIPC differs from local IPC in this regard. The

negative (2 cycles of RIPC) (352) and the positive findings (4 cycles of RIPC) (470) in rabbits could be explained by the presence of a threshold in this species.

The application of the RIPC stimulus on the limb is a much less risky and complicated approach than making internal organs ischaemic. Limb ischaemia using a tourniquet reduces ischaemic myocardial injury in the mouse and rat (262;297). Complete limb ischaemia, applied non-invasively to the hind limb, protects against reperfusion arrhythmias (368). More recently, our group demonstrated that 4 cycles of 5-minute hind limb ischaemia and reperfusion could reduce infarct size by 53% compared to control, in a porcine model of myocardial infarction *(figure 1.15)* (243). The protective effect is comparable to the one offered by "classic" ischaemic preconditioning, "intra-cardiac" and whole organ remote ischaemic preconditioning (399;538). In addition, intermittent limb ischaemia reduces respiratory dysfunction induced by ischaemia and reperfusion of the myocardium (524). Limb RIPC (3 cycles of 5 minutes ischaemia and reperfusion) has also been shown to be protective against ischaemia and reperfusion injury of remote tissues, other than the myocardium (7;265;342).

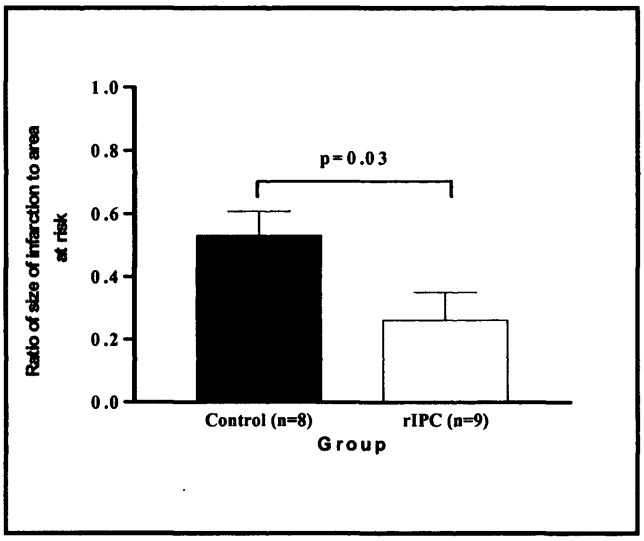


Figure 1.15 Original data demonstrating protection against myocardial IR injury by remote ischaemic preconditioning *(rIPC)* applied non-invasively on the hindlimb (Kharbanda *et al*, Circulation; 106: 2881, 2002).

1.4.2 Early and late protection by RIPC

Accumulating evidence from animal models of IR injury indicate that protection by RIPC may be "biphasic", with early and late windows of protection. This is similar to local ischaemic preconditioning, and suggest that local ischaemic preconditioning and RIPC may be different aspects of the same biological phenomenon.

Protection by RIPC is active immediately following the application of the RIPC stimulus. Induction of RIPC by short periods of ischaemia and reperfusion applied on the intestine, liver, kidney or limb *immediately before* IR, reduces injury in animal models of cardiac (423;520), renal (20), intestinal (120) and muscle (7) IR injury. Protection by RIPC lasts between 1 (267) and 3 hours (305). Similarly to early local ischaemic preconditioning, reversible modification of a pre-existing protein is a more

likely mechanism of early tissue protection from RIPC than alterations in protein expression.

Late protective effects of RIPC have been demonstrated in rat (510), mouse (478) and rabbit (471) models of myocardial infarction, in rat (266) and canine (303) models of skeletal muscle IR injury and rat models (37) of cerebral IR injury. The late protective effects by RIPC are evident 24 hours following the application of the RIPC stimulus on the intestine (510) or the limb (297) and last for up to 72 hours (525). Although there is evidence suggesting that late protection by RIPC might extend to up to 168 hours following the application of the RIPC stimulus (470), one study showed no protection 15 days after RIPC (372). For a summary of all studies to date on late RIPC see *Table A.9 (Appendix A)*.

It is not been definitively established whether there are two distinct phases of protection by RIPC (as for local ischaemic preconditioning) rather than a single prolonged period of protection. The time-course of protection by RIPC has not been described as studies have attempted to induce protection by administering RIPC stimuli either shortly before or 24-72 hours before IR. In order to establish that RIPC induces "biphasic" protection against IR, it would be necessary to demonstrate that the protective effects of RIPC disappear 3-4 hours after the application of the stimulus *(early or first window of protection)* and reappear 24 hours later, lasting for up to 72 hours *(late or second window of protection)*. Defining the time-course of protection by RIPC in animals and more importantly in humans, is fundamental for the optimum design of studies to investigate the potential of RIPC to limit IR injury in the clinical setting. Despite the uncertainties about the late protective effects of RIPC, the fact that

there is a prolonged interval between the application of the RIPC stimulus and the development of protection against IR allows for the production of new proteins that may be responsible for late protection by RIPC.

1.4.3 Mechanisms of RIPC

Compared to IPC, there is less information regarding the mechanisms and the exact signal transduction pathways of RIPC. Many of the same *trigger* and *mediator* candidates have been implicated and are currently under investigation. When considering preconditioning (direct or remote) and its associated mechanisms it is useful to distinguish between *triggers*, *mediators* and *end- effectors* (538). All parts of the preconditioning pathway that exert their activity only prior to the prolonged period of ischaemia (index ischaemia) are classified as triggers. Similarly, factors that exert their effect only after index ischaemia has begun can be classified as mediators. The end-effectors are responsible for the actual protective effect during the lethal ischaemic insult and/or the subsequent reperfusion period. For this reason, they could also be classified as mediators. In addition, a *memory element* could be present, somewhere between triggers and end-effectors, which is responsible for maintaining tissues in the preconditioned state (538).

1.4.3.1 Triggers of RIPC

Adenosine (467), bradykinin (423), endogenous opioids (120) and norepinephrine (368) have all been shown to trigger the protective effects of RIPC. The main approach used to demonstrate the involvement of the above factors in the initiation of the preconditioning signal is the administration of inhibitors, which act on receptors of interest, *before* the preconditioning stimulus is applied. The list of trigger factors

described below is not exhaustive. This is mainly because some of the factors that have been involved in mediating protection by RIPC have also been shown to trigger protection. For clarity, factors that have a mediator role will be described in the mediator section *(section 1.4.3.2)* and reference to their ability to also trigger RIPC will be made in the latter section as appropriate. *Table 1.1* provides a summary of studies to date demonstrating the role of various trigger factors in RIPC.

1.4.3.1.1 Adenosine

In the rabbit, pre-treatment with the non-selective adenosine inhibitor 8-(p-sulfophenyl) theophylline (8-SPT), abolished the protective effects of renal ischaemia (10 minutes) followed by reperfusion, applied immediately before a prolonged coronary artery occlusion (121;381;467). Activation of A_1 and A_3 adenosine receptors in ischaemic mesentery has been implicated in the rat (306). In the pig, 8-SPT had no effect on RIPC, but the dose might have been insufficient to fully block A_1 and A_3 receptors (7).

1.4.3.1.2 Bradykinin

Two studies (423;520) have investigated the potential role of bradykinin in rat models of myocardial infarction, with the RIPC stimulus was applied on the intestine. Antagonism of the bradykinin B_2 receptor with the selective inhibitor Icatibant (HOE-140) blocked cardioprotection induced by intra-mesenteric infusion of bradykinin (1μg/min). This suggests that there is a trigger role of bradykinin in this model of RIPC.

1.4.3.1.3 Opioids

The non-selective opioid receptor antagonist naloxone blocks remote preconditioning of the myocardium in the rat (378) and rabbit (117;120), as does 7-benzylidenenatrexone (BNTX) (513), consistent with a role for the δ_1 receptor.

1.4.3.1.4 Catecholamines

The role of catecholamines is unclear; reserpine (which depletes catecholamine stores) attenuates the protective effects of 10 minutes of limb ischaemia followed by 10 minutes of reperfusion in the rat (368). This is not mediated by α_1 adrenoreceptors, because administration of prazosin had no effect. Endogenous release of norepinephrine (NE) due to cerebral ischaemia failed to protect the rat myocardium against ischaemia and reperfusion injury, possibly because circulating levels are sub-threshold (109). It is possible that NE released locally at the site of preconditioning ischaemia acts synergistically with adenosine, bradykinin, and opioids to generate a protective signal.

1.4.3.1.5 Reactive oxygen species (ROS)

The anti-oxidant N-2- (mercaptopropionyl) glycine (MPG) administered before RIPC (15-minute infra-renal occlusion of the aorta) abolished protection in rats (513). The identity, source and mechanism of action of ROS are uncertain at present.

1.4.3.1.6 Triggers of late protection by RIPC; role of nitric oxide (NO)

Factors that trigger late protection by RIPC have not been identified. Although in local ischaemic preconditioning early and late phases of protection are induced by

similar triggers *(section 1.3)*, further studies are required to determine if this is also true in RIPC.

NO has been demonstrated to trigger late protection by ischaemic preconditioning by an eNOS-dependent mechanism with inducible NOS (iNOS) an end-effector of late ischaemic preconditioning (54). The end effector role of iNOS-derived NO in late protection by local and remote ischaemic preconditioning will be further discussed in *section 1.4.3.3.4.* In RIPC, NO is not necessary for early protective effects as protection by RIPC was not abolished by the NOS inhibitors Nω-nitro-L-arginine (L-NNA) and Nω-nitro-L-methyl-ester (L-NAME), when these were applied in advance of RIPC (265;389). However, in mice NO has an essential role in late protection by RIPC to reduce myocardial infarct size in ApoE/LDL receptor knockout animals (478).

RIPC Triggers								
Ref No	Adenosine	Bradykinin	Free Radicals	NO	NE	Opioids	KATP Channels	Prostaglandins
(305)	Rats		Rats					
(342)							Pigs (glibenclamide, 5-HD, HMR1098)	
(69)								Rats (SC560, rofecoxib, indomethacin)
(513)			Rats (MPG)			Rats (BNTX)		
(7)	Pigs (8-SPT, DPCPX)					Pigs (BNTX)		
(265)				Rats (L-NAME)				
(378)						Rats (Naloxone)		
(520)		Rats (HOE140)						
(306)	Rats (8-SPT, MRS-1191							
(120)						Rabbits (Naloxone)	Rabbits (glibenclamide)	
(109)					Rats			
(121)	Rabbits (8-SPT)							
(525)				Rats (L-NAME)				
(117)						Rabbits (Naloxone)		
(423)		Rats (HOE140)						
(118)	Rabbits				Rabbits			
(467)	Rabbits (8-SPT)							
(381)	Rabbits (8-SPT)						Rabbits (5-HD)	
(368)					Rats (reserpine, prazosin)			

Table 1.1 Summary of RIPC trigger factors. For each study, the animal type and agents used to demonstrate the trigger role of various factors *(in brackets)* are reported. In all studies pharmacological agents were administered in advance of the RIPC stimulus. Negative studies are in red.

1.4.3.2 Mediators of RIPC

As explained above, mediators of preconditioning (local or remote) are factors that exert their effects following the onset of the prolonged period of ischaemia that is responsible for tissue injury. Mediators are responsible for the transduction of the protective signal generated by the preconditioning stimulus and provide the link between trigger factors and end-effectors, which are ultimately responsible for inducing tissue protection against IR. Since mediators are important during index ischaemia, a factor involved in the mechanisms of RIPC can be classified as a mediator when an appropriate inhibitor administered *immediately before* or *during* index ischaemia can abrogate the protective effects of RIPC. *Table 1.2* summarises the putative mediators of RIPC.

1.4.3.2.1 Protein Kinase C (PKC)

Protein kinase C (PKC) is a serine/threonine kinase that is activated by lipid cofactors (diacylglycerol; DAG) derived from the breakdown of membrane lipids by phospholipase C (248). Several isoforms of PKC have been described in the heart and other tissues including the classic isoforms α, β and γ (activated by both DAG and calcium), the novel isoforms δ, η and ε (activated only by DAG) and the atypical isoform ζ (activation independent of DAG or calcium) (447). One of the prerequisites for activation of PKC is its translocation from the cytosolic to the particulate fraction (231;356). Upon activation, each of the PKC isoforms has the ability to "dock" on unique binding proteins known as receptors for activated C kinase (RACK) (338). The binding of PKC to the appropriate RACK's completes the activation of the isoform and enables the enzyme to phosphorylate any substrate that might be nearby (447). RACK's are located on the membrane of specific cellular organelles (e.g.

mitochondria) and as a result bring PKC isoforms into close proximity to specific substrate proteins, ultimately resulting to their phosphorylation (337). The specificity of PKC isoforms may be accounted for by their interaction with RACK's. The ε (169;308;391), δ (544) and α (508) isoforms of PKC have been implicated in the mechanisms of protection by local ischaemic preconditioning.

Administration of the PKC inhibitors chelerythrine (5mg/kg i.v.) or staurosporine (50μg/kg i.v.) 5 minutes prior to 30-minute coronary artery occlusion (index ischaemia) blocked the protective effects of RIPC (25 minutes of mesenteric artery occlusion followed by 15 minutes of reperfusion) in the rat (511). Although the specific PKC isoform involved was not determined in these studies, the schedule of inhibitor administration clearly demonstrates a mediator role of PKC in protection by RIPC in this model. In a further study, RIPC was shown to induce translocation of the ε isoform of PKC from the cytoplasmic to the particulate fraction, which is consistent with activation of the kinase in response to RIPC (520). Furthermore, administration of a selective inhibitor of the bradykinin B_2 receptor, that blocked protection by RIPC, prevented the distribution of PKCε to the particulate fraction, thus providing a link between PKC and a well characterised trigger of RIPC (520).

There are no data on the role of PKC in the late protection by RIPC, nor the involvement of other kinases, such as the tyrosine kinase family or mitogen activated protein kinases (MAPK), in early and late phases of RIPC.

1.4.3.2.2 Nuclear Factor Kappa-B (NFκB)

Nuclear factor kappa-B (NFκB) is a redox sensitive transcription factor that regulates a large number of genes involved in the inflammatory response such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) (162). The NFκB family consists of various members including p50, p52, p65 (RelA), c-Rel, and RelB (492). Members of the NFκB family form various homo- and heterodimers the most common of which is the p50 homodimer and the p52/RelA heterodimer (493). In their resting (inactive) state NFκB dimers can be found in the cytoplasm bound to inhibitory proteins known as IκB (88). In the presence of appropriate stimuli, NFκB inhibitory proteins are phosphorylated and then proteolytically degraded (89). This process results in NFκB activation and its translocation to the nucleus, where it binds to the promoter region of specific genes, initiating transcription (492).

NFκB has been shown to play a pivotal role in the pathophysiology of IR injury, as inhibition of its activation (340) or inhibition of the gene products resulting by its activation (such as leukocyte adhesion molecules, cytokines and chemokines) (76;180;296), protects the heart and other tissues against IR injury. However, NFκB is also involved in the mechanisms of the SWOP of ischaemic preconditioning, and inhibition of its activation and translocation to the nucleus inhibits late protection by ischaemic preconditioning (531). In the context of ischaemic preconditioning, NFκB is thought to be the common downstream pathway through which factors involved in late protection by ischaemic preconditioning (such as ROS and more importantly PKC) initiate expression of genes (e.g. antioxidant enzymes) that offer protection against IR (104;538). In addition, the activation of NFκB during preconditioning ischaemia could induce protection by inhibiting the deleterious activation of NFκB

during IR through an increase in the inhibitory proteins (IκB) that prevent the activation and translocation of NFκB (339;464). In this theory, NFκB activation during ischaemic preconditioning creates a negative feedback loop that prevents further activation of this transcription factor during IR.

A role for NFκB has been demonstrated in the mechanism of late protection by RIPC. Late protection by hindlimb RIPC against myocardial IR could not be induced in mice with homozygously targeted deletion of the NFκB p105 gene (297). p105 is the precursor of p50 (198), and these results demonstrated the crucial role of this NFκB subunit in the protective response by RIPC. In contrast to NFκB knockout animals, RIPC in wild-type mice was associated with activation and nuclear translocation of the p50 (but not the p65) subunit of NFκB 1-2 hours after the application of the RIPC stimulus that protected against myocardial IR 24 hours later. NFκB activation was observed in both the hind limb (site of preconditioning ischaemia) and the heart (site of index ischaemia). The same study also addressed the crucial question of how NFκB activation results in late protection by RIPC, and established a link between NFκB activation following RIPC and increased expression of iNOS in the myocardium 24 hours later, a response not observed in p105 knockout mice *(see section 1.4.3.4)* (297).

As far as the involvement of NFκB in the deleterious effects of IR is concerned, the study by Li *et al* provide strong evidence that NFκB activation during RIPC leads to protection by inhibiting NFκB activation during IR (297). In wild-type mice not subjected to hindlimb RIPC, IR resulted in a significant activation of NFκB in the heart, which was associated with IR induced injury. The latter was not observed in

wild-type mice subjected to RIPC (in this case NFκB activation was observed following RIPC), indicating that NFκB may induce protection via a negative feedback mechanism. Interestingly, NFκB knockout mice subjected to IR suffered less myocardial injury compared to wild-type animals, which is consistent with a dual role of this transcription factor in both the pathophysiology of IR and the mechanisms of late protection by RIPC. The involvement of NFκB in RIPC in humans remains to be confirmed.

1.4.3.2.3 ATP-sensitive Potassium (K_{ATP}) Channels

ATP-sensitive potassium (K_{ATP}) channels were first identified by Noma *et al* (361) in 1983. K_{ATP} channels are activated by a decrease in intracellular ATP-concentrations and are therefore normally inhibited by physiological levels of cellular ATP. They are modulated by various ligands (e.g. adenosine, acetylcholine), G-proteins, NO, fatty acids and pH (131;247). K_{ATP} channels are classified as either sarcoplasmic or mitochondrial and structurally consist of a sulphonylurea receptor (SUR) and an inwardly rectifying potassium (K_{ir}) pore channel subunit (175). Cardiac sarcoplasmic K_{ATP} ($sarcK_{ATP}$) channels are known to be composed of the SUR2A and Kir6.2 subunits (540). In contrast to the sarcolemmal subtype, mitochondrial K_{ATP} ($mitoK_{ATP}$) channels have not been cloned. Although $sarcK_{ATP}$ and $mitoK_{ATP}$ channels are thought to have similar structure, they have differential pharmacological responses, which have allowed the characterisation of the latter subtype (540). Agents specific for $mitoK_{ATP}$ channels include the blocker 5-hydroxydecanoate (5-HD), which inhibits the mitochondrial channels in the micromolar range (but not the sarcolemmal channels), and the opener diazoxide, which has been shown to be 1,000 times more potent in opening the mitochondrial than the sarcolemmal channels (158).

Opening of K_{ATP} channels has been consistently demonstrated in the mechanism of ischaemic preconditioning in various in vitro and in vivo models of IR injury. Gross *et al* (173), in a canine mode of myocardial infarction, were the first to demonstrate the role of these channels in cardioprotection by ischaemic preconditioning, as the non-specific K_{ATP} channel blocker glibenclamide blocked protection in this setting. A further 150 studies (in both animal and human models of IR) of K_{ATP} channels have mostly demonstrated that opening of these channels is a prerequisite for the development of protection by ischaemic preconditioning. Although it was initially believed that sarcK_{ATP} channels mediated protection by ischaemic preconditioning (174), accumulating evidence point towards mitoK_{ATP} channels as the key factor in the mechanisms of this phenomenon (157;311). However, a combined role of both sarcK_{ATP} and mitoK_{ATP} cannot be excluded (461). *(For a summary of studies on the role of K_{ATP} channels in local and remote ischaemic preconditioning see tables B.1 to B.6, Appendix B).*

Controversies remain regarding the exact position of these channels in the signal transduction pathway of ischaemic preconditioning (538). The majority of studies support a mediator or end-effector role and this has been demonstrated by administering K_{ATP} channel blockers *immediately before* or *during* index ischaemia. However, recent experiments have re-examined this assumption. Administration of 5-HD *during* the application of the preconditioning stimulus blocks protection, which is consistent with K_{ATP} channels acting to trigger ischaemic preconditioning (370;507). Other groups have shown that mitoK_{ATP} channel opening induces protection by activating downstream kinases (including PKC and tyrosine kinases), which further supports a trigger role for these channels (366;509). The intriguing question is how

channel opening induces kinase activation. It has been proposed that opening of mitoK_{ATP} channels leads to the production of free radicals by the mitochondria (probably by site III of the electron transport chain), which then mediate the activation of downstream kinases (144;261). The latter theory was further supported by Cohen *et al* (93) who showed that G_i-protein receptors involved in preconditioning (with the exception of adenosine receptors) couple through a mitoK_{ATP}/free radical pathway. Consequently, the current weight of evidence supports both a trigger and a mediator role of K_{ATP} channels. It is possible that K_{ATP} channel opening triggers a free radical mediated kinase cascade which in turn acts by a positive feedback mechanism to keep the channel open during index ischaemia and thus mediate protection (538).

K_{ATP} channels have also been shown to be involved in the protective mechanisms of RIPC. In a rabbit model of myocardial infarction, the selective mitochondrial K_{ATP} channel inhibitor 5-HD (5mg/kg i.v.) blocked the infarct reducing effects of RIPC *(figure 1.16)* (381). In this study RIPC was induced by 10 minutes of renal ischaemia followed by 10 minutes of reperfusion applied immediately prior to myocardial IR. However 5-HD was applied prior to the RIPC stimulus and as a result it was not clear whether K_{ATP} channels acted as a trigger or mediator or RIPC.

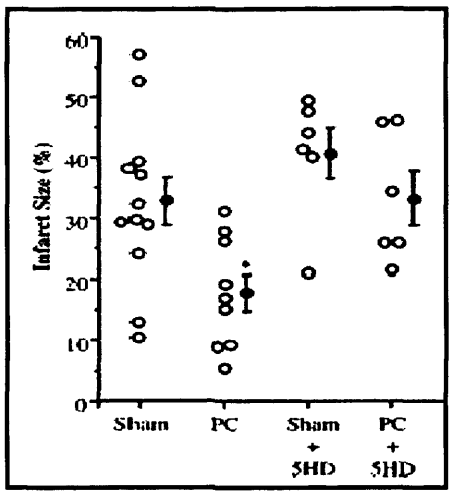


Figure 1.16 Original data demonstrating the role of mitochondrial K_{ATP} channels in the mechanisms of myocardial protection by RIPC (Pell *et al*, Am J Physiol Heart Circ Physiol; 44:1542, 1998). *PC: preconditioned (remote ischaemic); 5-HD: 5-hydroxydecanoate.*

Subsequently, the mitoK_{ATP} channel was further implicated in the protection by RIPC, as 5-HD (5mg/kg i.v.) abrogated the protective effects of mesenteric ischaemia when administered prior to the onset of myocardial ischaemia (511).

Mitochondrial K_{ATP} channels have also been implicated in the *memory* element of RIPC. Hindlimb RIPC in pigs induced a preconditioned state in the myocardium even when the heart was explanted and subsequently made ischaemic for a period of 45 minutes. Protection was not evident when glibenclamide (10μM) or 5-HD (100μM) was added after the heart was explanted prior to IR (262). These experiments, in addition to demonstrating the role of mitoK_{ATP} channels as mediators of RIPC also provide the first evidence on how the protective signal (generated in a remote site) could be memorized by the tissues; protection against IR could be maintained for as

long as the mitoK_{ATP} channels remained open and would disappear when the channels returned to their resting (closed) state. However, further studies would be required to confirm such a role for K_{ATP} channels in RIPC.

Although there is strong evidence that mitoK_{ATP} channels mediate protection by RIPC against IR injury, some studies have also demonstrated that the channels are also involved in triggering protection by RIPC (120;342), because blocking the channels during the application of the RIPC stimulus inhibits the protective effects of RIPC. Thus, it seems that that the dual role of K_{ATP} channels, described for local ischaemic preconditioning, is also evident in RIPC. Exactly how K_{ATP} channels trigger RIPC is not clear, but it is possible that a mitoK_{ATP}/ROS pathway could be involved (513). No evidence is currently available on the role of K_{ATP} channels in late protection by RIPC. However, given the similarities between local ischaemic preconditioning and RIPC, it is very likely that these channels could mediate the late protective effects of RIPC. In addition, there are no studies demonstrating a role of K_{ATP} channels in RIPC in humans.

RIPC mediators				
Ref No	**PKC**	**KATP Channels**	**NFkB**	**Prostaglandins**
(297)			Mice (p105 gene deletion)	
(262)		Rats (glibenclamide, 5-HD, HMR1098		
(342)		Pigs (glibenclamide, 5-HD, HMR1098)		
(69)				Rats (SC560, rofecoxib, indomethacin)
(6)		Pigs (5-HD)		
(511)	Rats (chelerythrine, staurosporine)	Rats (5-HD)		
(512)	Rats (chelerythrine)			
(520)	Rats (chelerythrine)			
(120)		Rabbits (glibenclamide)		
(381)		Rabbits (5-HD)		

Table 1.2 Summary of studies on mediators of RIPC. For each study, the animal type and agents used to demonstrate the mediator role of various factors *(in brackets)* are reported. In all studies pharmacological agents were administered in advance or during index ischaemia.

1.4.3.3 End-effectors of RIPC

Despite extensive research carried out since the original description of ischaemic preconditioning in 1986 (346), the specific *end-effector(s)* (the factor(s) responsible for the development of protection during ischaemia), has not been determined. Some of the factors that have been involved as mediators of ischaemic preconditioning have also been proposed to be end effectors of the phenomenon (538). Conclusions about the potential end-effectors of RIPC can be drawn by assuming that local ischaemic preconditioning and RIPC are mechanistically similar. Although there is evidence to support this, important differences may exist in the end-effectors of local and remote ischaemic preconditioning.

In this section, I will mainly focus on end-effectors that have been shown to be part of the distal signal transduction pathway of RIPC and present supporting data. I will also summarise factors that have been implicated as end-effectors of local ischaemic preconditioning and as a result may also be involved in inducing protection by RIPC, although definitive proof for such a role is currently lacking.

1.4.3.3.1 Metabolic effects

The oldest theory about the end effector of ischaemic preconditioning was introduced by Murry (348) who showed that that ischaemic preconditioning attenuated the reduction in ATP in the myocardium during ischaemia and reperfusion. Since then, the improvement in tissue energetics caused by ischaemic preconditioning has been confirmed by several other studies (538). However the effect of preconditioning to preserve ATP during ischaemia is not sufficient to compensate for the effect of the preconditioning ischaemia on ATP, so that there remains an overall deficit in tissue

ATP (257). In RIPC, preconditioning has no effect on baseline tissue ATP in advance of prolonged ischaemia. Preservation of high-energy phosphates has therefore been implicated as an end effector of RIPC. RIPC protects against skeletal muscle IR injury in dogs, and preserves ATP levels (303). No differences in energy utilisation during reperfusion were observed between control and RIPC groups, demonstrating that energy preservation during ischaemia was the key determinant of the increased tissue viability observed in the RIPC group. These observations were subsequently confirmed in a rabbit model of myocardial infarction (467). Using ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopy, RIPC applied on the kidney prior to myocardial IR resulted in significant attenuation in the decrease of ATP and phosphocreatine (PCr) during ischaemia. This effect was similar to that of local ischaemic preconditioning and was attenuated by the adenosine receptor blocker SPT (which also blocked the infarct sparing effect of RIPC). RIPC also resulted in better recovery of ATP and PCr during reperfusion. The energy sparing effects of RIPC have also been recently demonstrated in a porcine model of skeletal muscle infarction. RIPC, induced non-invasively by short periods of ischaemia and reperfusion applied on the limb, preserved skeletal muscle ATP and reduced lactate concentration during ischaemia (7). RIPC also improved recovery of metabolic balance following reperfusion. Whether RIPC decreases ATP utilisation or degradation during ischaemia remains to be determined.

RIPC also attenuates the decrease in intracellular pH (pHi) observed during ischaemia (467). This may be secondary to preservation of ATP and reduced utilisation of anaerobic pathways for energy production (389) which would result in reduced generation of lactate. The preservation of high-energy phosphates and pHi by RIPC

could also offer protection by preventing osmotic swelling and failure of the extracellular membrane.

1.4.3.3.2 ATP-sensitive Potassium (K_{ATP}) Channels

As has been described in *section 1.4.3.2.3*, K_{ATP} channels have been involved in both the trigger and mediator mechanisms of local and remote ischaemic preconditioning. However, the requirement for opening of these channels for the induction of the protective effects of preconditioning is consistent with an end-effector role in preconditioning.

How the opening of these channels results in the protection observed during ischaemia is not clear. Mitochondrial K_{ATP} channels might provide protection by decreasing the uptake of Ca^{2+} by the mitochondria during IR (208). The IR-induced uptake of Ca^{2+} by the mitochondria opens the mPTP during reperfusion, disrupting mitochondrial function and leading to cell injury (184). Opening of mitochondrial K_{ATP} channels reduces Ca^{2+} entry, preventing opening of the mPTP and protecting against IR injury (195). The mPTP channel opener actractyloside blocks diazoxide-induced preconditioning, consistent with the mPTP acting downstream from the K_{ATP} channel. In addition, late protection against IR induced by adenosine (a well-characterised trigger of local and remote ischaemic preconditioning acting via the opening of K_{ATP} channels) is mediated by inhibition of the opening of mPTP, further supporting a role of a K_{ATP} channel-mediated inhibition of mPTP opening in ischaemic preconditioning (195).

Opening of the mitochondrial K_{ATP} channels causes K^+ to enter the mitochondria, and a (small) increase in mitochondrial volume (156). This might preserve the functional coupling of mitochondrial creatine kinase and adenine nucleotide translocase (ANT), and result in increased production of cytoplasmic PCr (126;272). The latter could explain the preservation by RIPC of PCr levels during ischaemia observed in a rat model of myocardial infarction (467). Moreover, functional coupling of creatine kinase and ANT decreases permeability of mitochondrial membranes to adenine nucleotides which, in addition to further facilitating PCr production, also leads to decreased ATP breakdown by the mitochondria.

The above studies support an end effector role for the mitochondrial K_{ATP} channels. A role for the sarcolemmal K_{ATP} channels has been suggested in the late protective effects of ischaemic preconditioning in the myocardium (44). Myocardial IR is associated with profound functional impairment of the previously ischaemic myocardium, a phenomenon known as "stunning"(56). The anti-stunning effects of late ischaemic preconditioning may be due to shortening of action potential duration in cardiac myocytes, mediated by opening of the sarcolemmal K_{ATP} channels; late protection of stunning has been observed with non-specific K_{ATP} channel blockers but not mitochondrial K_{ATP} channel-specific inhibitors (174;466). The potential mechanisms through which preconditioning ("local" or remote) induced K_{ATP} channel opening may result in protection against ischaemia-reperfusion injury are summarised in *figure 1.17.*

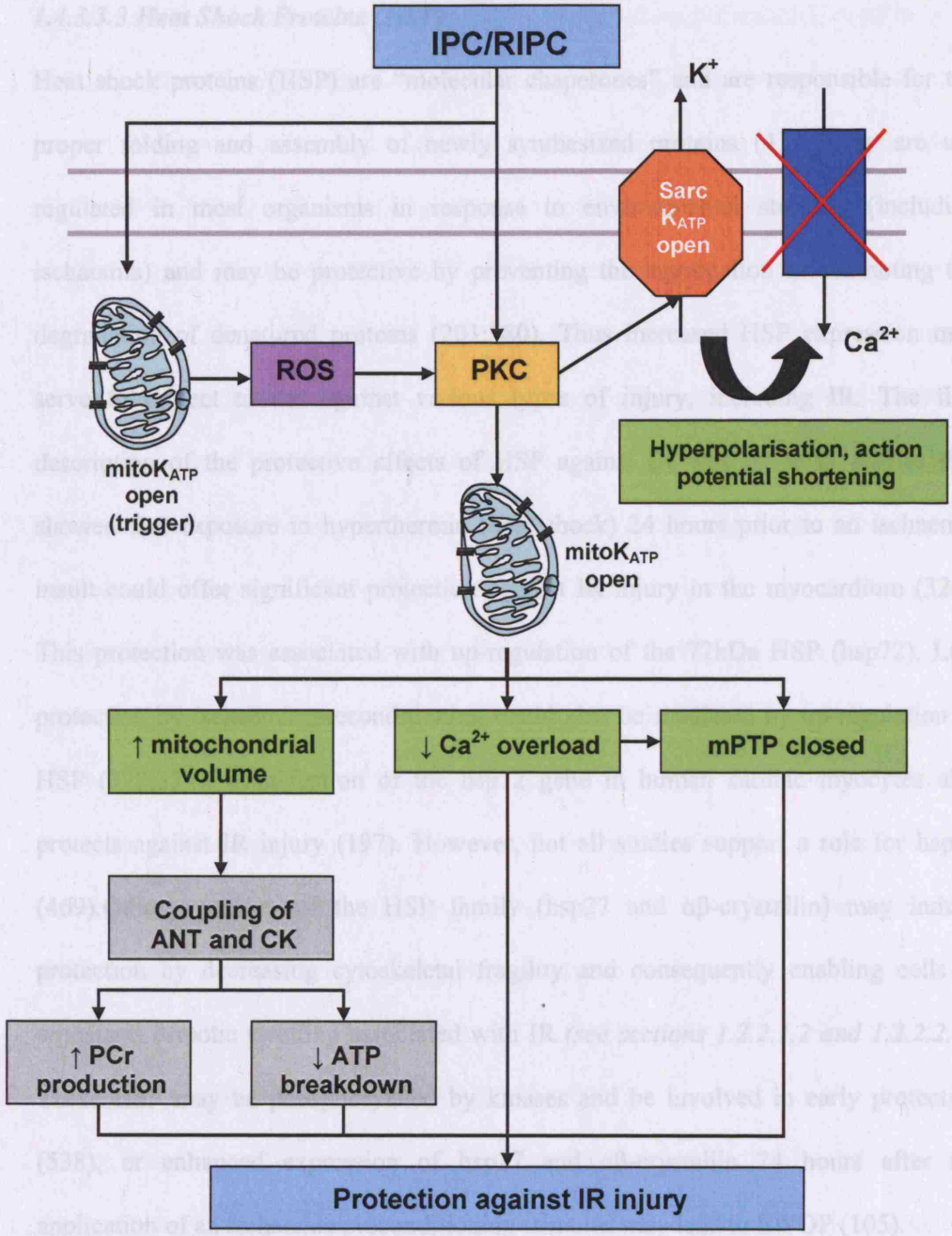


Figure 1.17 Mechanisms of protection against IR injury by preconditioning-induced K_{ATP} channel opening. Adapted from Gross *et al*, Circ Res; 84: 973, 1999. *Sarc/mitoK_{ATP}: sarcolemmal/mitochondrial K_{ATP} channel; ROS: reactive oxygen species; ANT: adenine nucleotide translocase; CK: creatine kinase; PCr: phosphocreatine.*

1.4.3.3.3 Heat Shock Proteins (HSP)

Heat shock proteins (HSP) are "molecular chaperones" and are responsible for the proper folding and assembly of newly synthesized proteins (41). They are up-regulated in most organisms in response to environmental stresses, (including ischaemia) and may be protective by preventing the aggregation or promoting the degradation of denatured proteins (201;380). Thus increased HSP expression may serve to protect tissues against various types of injury, including IR. The first description of the protective effects of HSP against IR was made in studies that showed that exposure to hyperthermia (heat shock) 24 hours prior to an ischaemic insult could offer significant protection against IR injury in the myocardium (324). This protection was associated with up-regulation of the 72kDa HSP (hsp72). Late protection by ischaemic preconditioning could also be mediated by up-regulation of HSP (322;323). Transfection of the hsp72 gene in human cardiac myocytes also protects against IR injury (197). However, not all studies support a role for hsp72 (469).Other members of the HSP family (hsp27 and αβ-crystallin) may induce protection by decreasing cytoskeletal fragility and consequently enabling cells to withstand osmotic swelling associated with IR *(see sections 1.2.2.1.2 and 1.2.2.2.1)*. These HSP may be phosphorylated by kinases and be involved in early protection (538), or enhanced expression of hsp27 and αβ-crystallin 24 hours after the application of an ischaemic preconditioning stimulus may lead to SWOP (105).

Increased expression of HSP can also be observed in tissues remote from those undergoing preconditioning, supporting a role of HSP as end-effectors of RIPC. The first evidence of up-regulation of HSP in response to sublethal ischaemia was reported two years before the concept of RIPC was introduced. Short periods of ischaemia and

reperfusion in one area of the rabbit heart resulted in significant up-regulation of hsp72 in remote myocardium not subjected to ischaemia, which was evident as early as two hours and even more striking at 24 hours after ischaemia (255). RIPC applied 48 hours before IR induced ischaemic protection and up-regulation of hsp72 in skeletal muscle (but not hsp27, 60, 90) (303). These observations were subsequently confirmed in a rabbit model of myocardial ischaemia (470). Ischaemic preconditioning applied on the left ventricular myocardium induced increased expression of hsp72 in both the myocardium undergoing preconditioning and remote "virgin" myocardium. Up-regulation of hsp72 was detected 3 hours following the application of the preconditioning stimulus and was most prominent after 24 and 48 hours, decreasing thereafter. In conclusion, the latter studies support a role for HSP (mainly hsp72) as end-effectors of late protection by RIPC. The enhanced expression of these proteins may induce protection against IR by the mechanisms described above for local ischaemic preconditioning. However, the relative importance of HSP in late local and remote ischaemic preconditioning is yet to be determined and more work needs to be undertaken to define their precise role.

1.4.3.3.4 Inducible Nitric Oxide Synthase (iNOS)

As described in *section 1.4.3.1.6*, nitric oxide (NO) has been implicated as both a trigger factor and possibly the end-effector of late ischaemic preconditioning. According to Bolli's NO hypothesis of ischaemic preconditioning, eNOS-derived NO triggers the SWOP, and protection is then mediated by NO that is produced by inducible nitric oxide synthase (iNOS), the expression of which is up-regulated 24 hours following the application of the ischaemic preconditioning stimulus (54). Delayed protection against IR can be blocked pharmacologically by selective iNOS

inhibitors administered during index ischaemia (217;465) and cannot be induced in iNOS knock out animals (182;545).

Enhanced NO production has been shown to protect against IR by reducing Ca^{2+} overload in the myocardium following IR, by stimulating myocardial glycolysis/ATP production and preserving the metabolic balance during ischaemia, by reducing myocardial oxygen consumption and by attenuating the inflammatory response to IR (427). Another theory suggests that elevated NO levels, generated as a result of iNOS induction, modify the opening characteristics and the activity of K_{ATP} channels. Such a view, which is more consistent with a mediator rather than an end-effector role for iNOS in the SWOP, is indirectly supported by evidence that NO stimulated cGMP activates PKG (a cGMP dependent protein kinase) (365), which causes K_{ATP} channel activation and induction of protection by the mechanisms described previously *(section 1.4.3.3.2)*.

Regarding RIPC, activity of iNOS is significantly increased 24 hours following the application of the RIPC stimulus, which is consistent with increased expression of the enzyme (510). Administration of the iNOS inhibitors aminoguanidine (300mg/kg, s.c.) or S-methylisothiourea sulphate (SMT; 3mg/kg, i.v.), blocked late protection of RIPC against myocardial infarction (510). The protective effects of increased NO production by iNOS in this model were attributed to NO-induced attenuation of the inflammatory response to IR (reduced neutrophil infiltration). In mice, late protection by limb RIPC was associated with a significant increase in the expression of iNOS (dependent on NFκB), and such protection could not be observed in iNOS knockout mice (297). Based on the above, it can be suggested that PKC-mediated activation of

NFκB (induced by triggers of RIPC; *see section 1.4.3.2.2*), results in transcriptional activation of the iNOS gene and increased expression of the enzyme, which leads to the late protective effects by RIPC.

1.4.3.3.5 Cyclooxygenase (COX)

Another proposed end effector of the SWOP of ischaemic preconditioning is cyclooxygenase 2 (COX-2) (58;538). This is somewhat surprising, since COX-2 has been considered to have pro-inflammatory and thus detrimental effects in cardiovascular and other tissues (497). However, recent evidence demonstrates that COX-2 and its products (e.g. PGE_2, PGI_2) have a central role in the mechanisms of the SWOP of ischaemic preconditioning (58). Bolli's group observed that ischaemic preconditioning results in the up-regulation of COX-2 in the myocardium 24 hours following the application of the protective stimulus and that this enzyme is somehow responsible for the protective effects of SWOP (442). In addition, pharmacological activation of δ_1 opioid receptors was shown to induce late preconditioning-like protection against IR via a COX-2 mechanism (256), further supporting a key role of this enzyme in SWOP. Exactly how COX-2 up-regulation results in protection against IR is not currently known, but it seems that the enhanced expression of COX-2 is mediated by a PKC, NFκB pathway (58;530). Interestingly, COX up-regulation and COX-mediated late protection seems to be dependent on iNOS derived NO, as iNOS inhibition abrogated COX-mediated SWOP (via a cGMP/PKG mediated pathway) (443).

There is currently no direct evidence supporting an end effector role for COX-2 in late RIPC. In a model of gastric IR, COX-1/COX-2 produced PGE_2 has been

demonstrated in the mechanisms of early RIPC, with a significant increase in COX-2 mRNA levels in gastric tissue shortly after cardiac or hepatic RIPC (70). Such rapid up-regulation in COX-2 mRNA levels could result in increased protein production 24 hours after the application of the preconditioning stimulus and subsequent COX-2 mediated late protection by RIPC. It is possible that increase in cardiovascular events in patients taking COX-2 inhibitors reflects an anti-preconditioning effect.

1.4.3.4 Transfer of protection to remote tissues

One of the most intriguing questions regarding RIPC is how the protective signal, generated in the tissue/organ undergoing preconditioning, is transferred to remote tissues. This aspect of RIPC has been the focus of a large proportion of the studies investigating the phenomenon and currently there are three main theories *(figure 1.18)*, supported by strong experimental evidence, which provide plausible answers to the above question. In this section I will summarise the evidence that supports each of these theories.

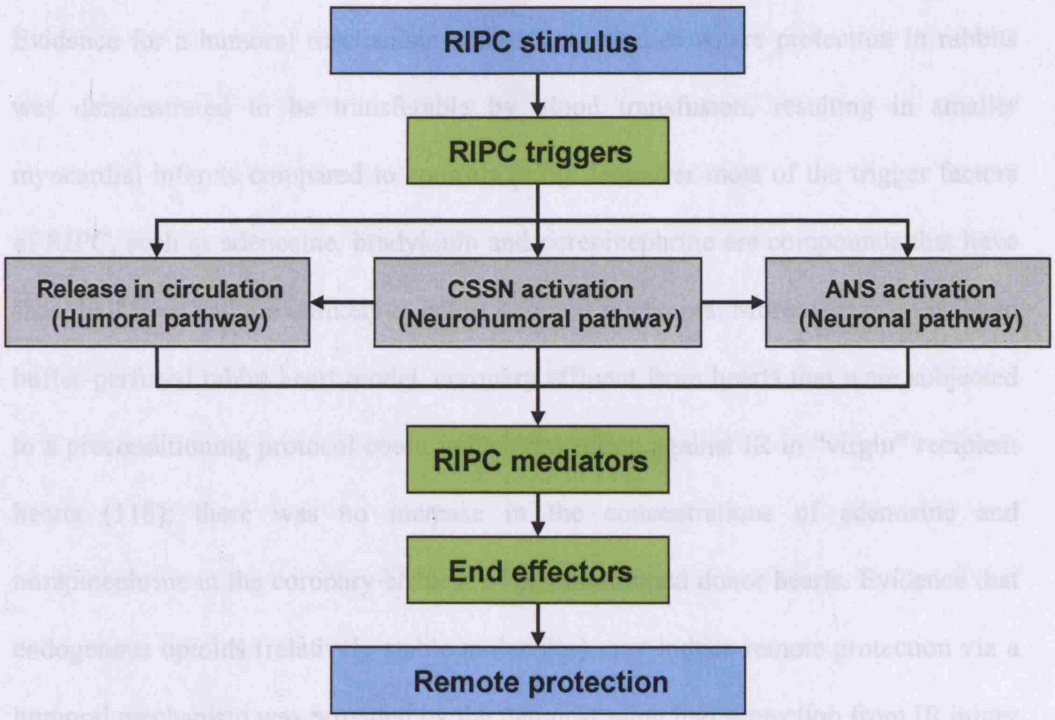


Figure 1.18 Proposed mechanisms for the transfer of the protective signal, generated by the application of the RIPC stimulus, to remote tissues.

1.4.3.4.1 Humoral theory

This first theory suggests that the short periods of ischaemia and reperfusion that constitute the RIPC stimulus results in the release of a compound that is then transported by the circulation and "triggers" the protective state in distant tissues. This explanation for the systemic effects of ischaemic preconditioning is very appealing as the identification of such a compound could provide novel pharmacological therapies that may induce a "whole body" preconditioned state with obvious clinical implications.

Evidence for a humoral mechanism comes from studies where protection in rabbits was demonstrated to be transferable by blood transfusion, resulting in smaller myocardial infarcts compared to controls (119). However most of the trigger factors of RIPC, such as adenosine, bradykinin and norepinephrine are compounds that have short half lives and are unlikely to act as humoral mediators. Moreover, in an isolated buffer-perfused rabbit heart model, coronary effluent from hearts that were subjected to a preconditioning protocol could induce protection against IR in "virgin" recipient hearts (118); there was no increase in the concentrations of adenosine and norepinephrine in the coronary effluent of preconditioned donor hearts. Evidence that endogenous opioids (relatively stable molecules) may induce remote protection via a humoral mechanism was provided by the demonstration that protection from IR injury caused by coronary effluent from preconditioned rabbit hearts, was blocked by the non-selective opioid antagonist naloxone (2μM) (117). Moreover raised concentrations of the endogenous opioids met- and leu-enkephalin were found in the coronary effluent from donor hearts subjected to preconditioning. However, the concentration of endogenous opioids from preconditioned donor hearts was insufficient to reduce IR-induced myocardial injury, demonstrating that a "cocktail" of hydrophobic compounds (including endogenous opioids) is probably responsible for the protection observed (117). The abrogation of protection induced by administration of naloxone can be explained by the fact that blocking the opioid component of the synergistic hydrophobic "cocktail" may weaken the protective signal and render the preconditioning stimulus "sub-threshold".

Additional evidence supporting the humoral theory of RIPC and the important role of endogenous opioids in the transfer of protection have been provided by Dickson *et al*

in a recent study in a rabbit model of intestinal IR injury (120). They demonstrated that coronary effluent from donor hearts subjected to preconditioning could prevent IR injury in isolated rabbit jejunal segments and that this protection could be blocked by naloxone and the K_{ATP} channel blocker glibenclamide. This study also supports a role of endogenous opioids in the transfer of the protective signal to distant sites, and establishes a link between such a humoral mediator and best characterised mediator/end-effector of ischaemic preconditioning, the K_{ATP} channel. Additional candidate factors cannot be excluded; when the production of prostaglandins was blocked in donor preconditioned animals, the protective effects of coronary effluent were lost (120).

In summary, spread of protection from the site of preconditioning to remote tissues may involve endogenous opioids, prostaglandins and other still unidentified (hydrophobic) compounds produced during preconditioning ischaemia. They are likely to be released in the circulation during the reperfusion phase of the preconditioning stimulus, and activate end effectors (e.g. K_{ATP} channels). Such a theory could also explain the requirement for a RIPC stimulus to include periods of reperfusion in between the short periods of preconditioning ischaemia (161;328;512); lack of reperfusion would prevent the release of the humoral factors in the circulation and as a result protection of remote tissues against IR would not be achieved. *(For a summary of studies supporting the humoral theory of RIPC see table 1.3).*

1.4.3.4.2 Neuronal theory

The second theory has implicated neuronal mechanisms and more specifically the autonomic nervous system (ANS) *(figure 1.19)* (520). Interruption of the cholinergic transmission in the autonomic ganglia by the nicotinic cholinergic antagonist hexamethonium (20mg/kg, i.v.) blocked the infarct sparing effect of a RIPC stimulus applied on the mesenteric vascular bed in rats (161). Hexamethonium did not have an effect on local ischaemic preconditioning against myocardial IR injury in the same model. Protection against IR was not observed when periods of preconditioning ischaemia were not followed by reperfusion which suggested that a factor produced during ischaemia and released during reperfusion may be responsible for activating the neuronal pathway. Similarly, hexamethonium blocked RIPC in the rat (induced by mesenteric ischaemia); intramesenteric infusion of bradykinin in advance of IR also reduced myocardial injury and this was blocked by pre-treatment with hexamethonium (423). This, together with the observation that the protective effects of RIPC could be abrogated by the bradykinin receptor antagonist HOE-140, suggests that local release of bradykinin following the application of the RIPC stimulus activates the ANS to mediate the remote protective effects. The adenosine receptor inhibitor 8-SPT also blocked the protective effects of intestinal RIPC and intra-mesenteric infusion of adenosine (306). RIPC of the kidney was shown to be dependent on intact renal nerves, and could be prevented by adenosine blockade (121).

Not all studies have demonstrated that hexamethonium blocks RIPC (7;511;512). Whether this is attributed to differences in the experimental models used is not clear, but it is possible that involvement of neuronal and humoral factors might result in

there being sufficient redundancy to make RIPC resistant to blockade of one system. The role of the autonomic nervous system in human RIPC has not been determined *(For a summary of studies on the role of ANS in RIPC see table 1.3)*.

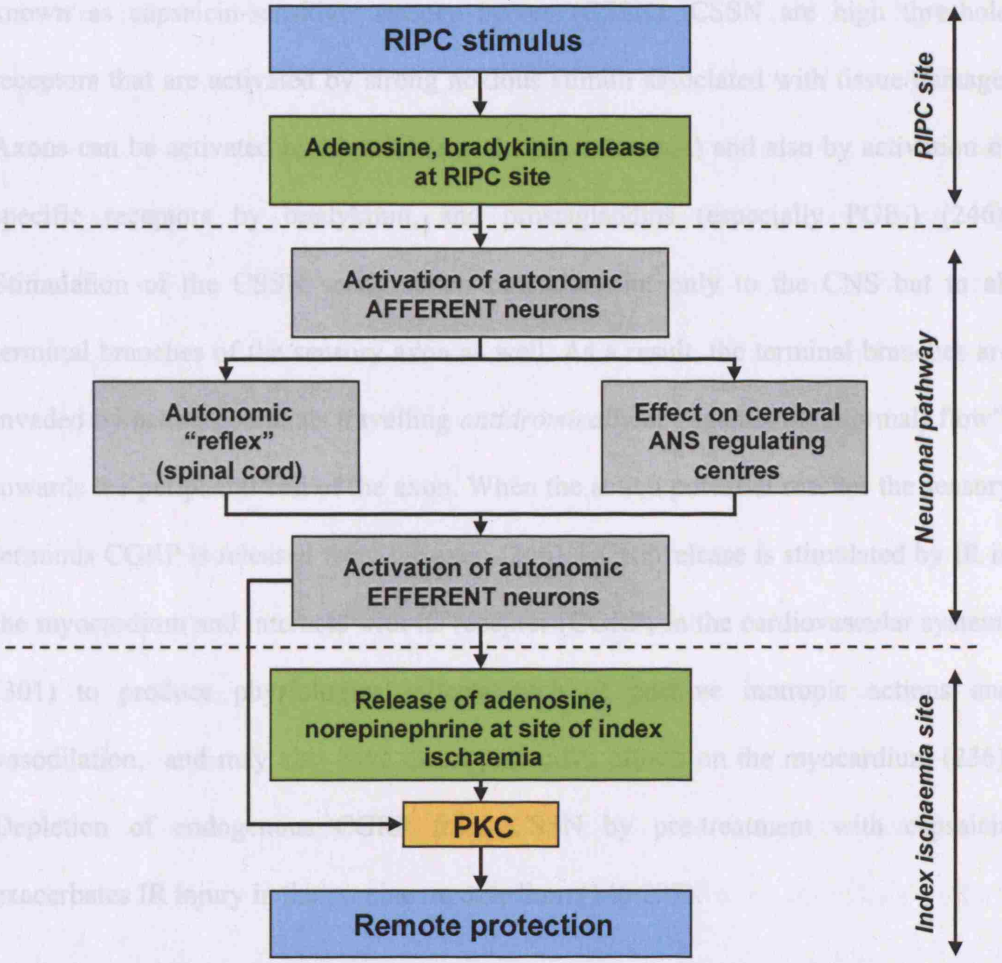


Figure 1.19 Role of the autonomic nervous system *(ANS)* in the transfer of the protective signal from the site of RIPC stimulus application to remote tissues (neuronal pathway).

1.4.3.4.3 Capsaicin-Sensitive Sensory Nerves and Calcitonin Gene-Related Peptide; Potential Role of a Neurohumoral Pathway in the Mechanisms of RIPC

Calcitonin gene-related peptide (CGRP) is a major neuropeptide in C-fibres (nociceptors) (301). These sensory fibres are sensitive to capsaicin and they are known as capsaicin-sensitive sensory nerves (CSSN). CSSN are high threshold receptors that are activated by strong noxious stimuli associated with tissue damage. Axons can be activated by high K^+ (e.g. during ischaemia) and also by activation of specific receptors by bradykinin, and prostaglandins (especially PGE_2) (246). Stimulation of the CSSN sends action potentials not only to the CNS but to all terminal branches of the sensory axon as well. As a result, the terminal branches are invaded by action potentials travelling *antidromically* or "against the (normal) flow", towards the peripheral end of the axon. When the action potential reaches the sensory terminus CGRP is released from the axon (246). CGRP release is stimulated by IR in the myocardium and interacts with its receptor ($CGRP_1$ in the cardiovascular system) (301) to produce physiological effects such as positive inotropic actions and vasodilation, and may also have direct protective effects on the myocardium (236). Depletion of endogenous CGRP from CSSN by pre-treatment with capsaicin exacerbates IR injury in the porcine myocardium (146;237).

CGRP has also been involved in the mechanisms of ischaemic preconditioning (142;302;315). It is released following the application of an ischaemic preconditioning stimulus (238;315;547), and inhibition of CGRP activity (by desensitisation of CSSN using high-dose capsaicin or selective inhibition of the $CGRP_1$ receptor) abolished the protective effects of ischaemic preconditioning (142;547). Endogenous NO has also been shown to modulate the release of CGRP

and it is possible that some of the protective effects of the increased NO production (due to iNOS up-regulation) observed in the late phase of ischaemic preconditioning are mediated by CGRP (212;301).

Similarly, RIPC applied to the mesentery increases release of CGRP, and high-dose capsaicin denervation blocked tissue protection (471). As for local IPC, CGRP is involved in early and second window protection from RIPC, and may involve NO generation (525). Increased levels of CGRP (a-CGRP) mRNA have been reported in the dorsal root ganglia of preconditioned rats (213). COX-derived prostaglandins (PGE_2) stimulate CGRP-mediated protection, as COX inhibition reduced protection from RIPC and reduced CGRP release (69).

Based on current evidence, a neurohumoral mechanism of RIPC can be summarised as in *(figure 1.20)*. The application of the RIPC stimulus induces the release of nociceptive stimulants (such as bradykinin and PGE_2) which results in the activation of CSSN at the site of preconditioning. CSSN activation is accompanied by release of CGRP that can then be transported by the circulation to distant sites and induce protection. The protective effects of CGRP can be attributed to its vasodilatory effects or to direct effects to the myocardium and other tissues mediated by G-protein coupled receptors (301). CGRP has been shown to activate PKC (383) and may induce protection by activating K_{ATP} channels (516) or by attenuating the inflammatory response to IR (332;384). In addition to this, CSSN activation may result in ANS activation and CGRP may also be involved in the modulation of this response (71). CSSN-mediated ANS activation in RIPC may result in protection by enhancing the ability of the myocardium or other distant tissues to release endogenous

CGRP during IR. Thus, it is possible that the release of adenosine at distant sites by ANS activation due to RIPC results in protection by increasing the release of CGRP following IR (432).

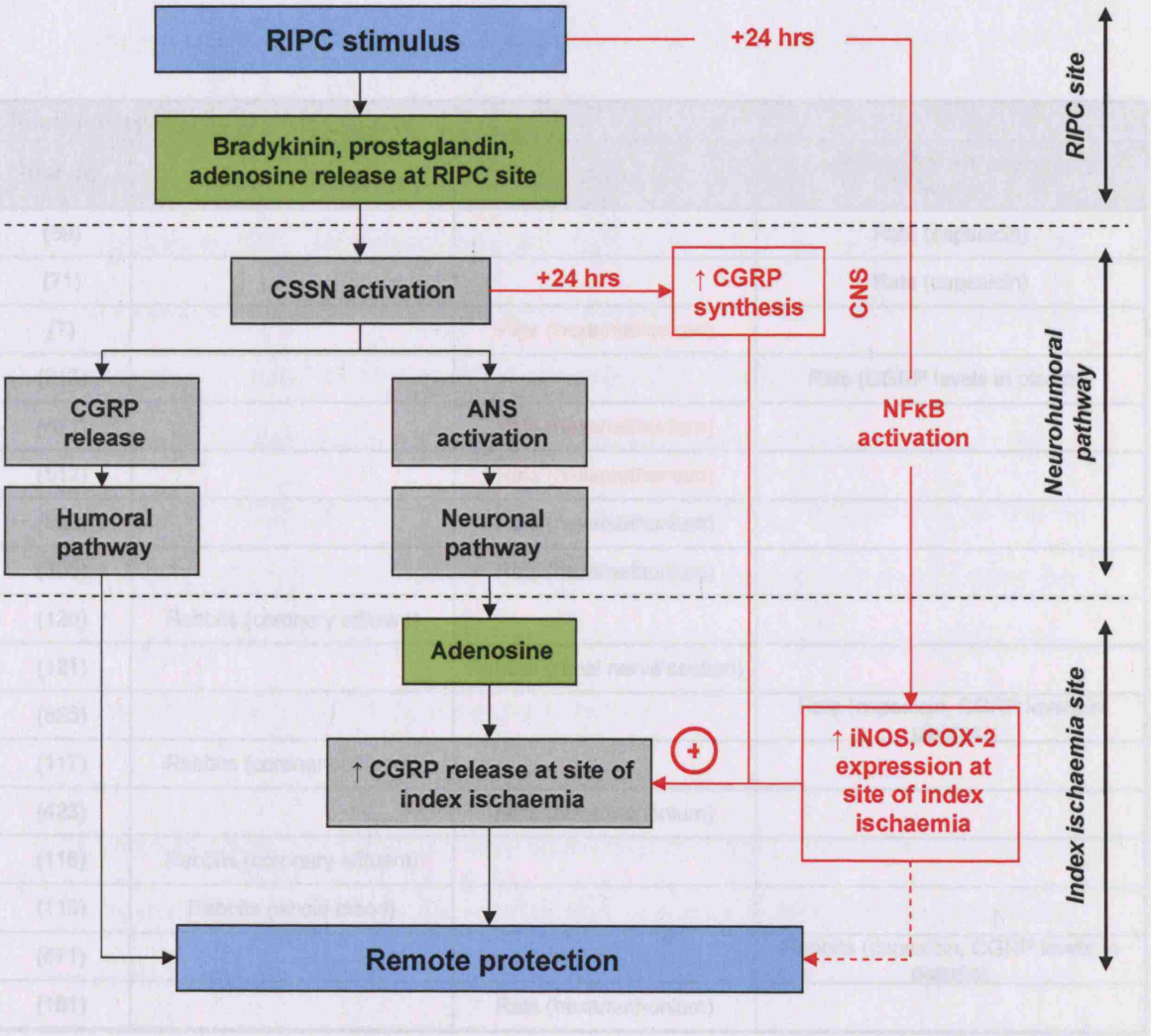


Figure 1.20 Interaction of neuronal and humoral pathways (neurohumoral pathway) in the mechanisms of transfer of protection by RIPC to distant tissues. The neurohumoral pathway has also been implicated in the mechanisms of delayed RIPC *(in red). CSSN: capsaicin-sensitive sensory nerves; CGRP: Calcitonin gene-related peptide; ANS: autonomic nervous system; CNS: central nervous system.*

Table 1.3 provides a summary of the studies investigating the role of CSSN in RIPC. It also summarises studies demonstrating a role for the other two pathways (humoral and neuronal/ANS-mediated) in the transfer of the RIPC protective signal to remote tissues.

Transfer of protection to remote tissues			
Ref No	**Humoral Factor**	**ANS**	**Capsaicin Sensitive Sensory Nerves**
(69)			Rats (capsaicin)
(71)			Rats (capsaicin)
(7)		Pigs (hexamethonium)	
(213)			Rats (CGRP levels in plasma)
(511)		Rats (hexamethonium)	
(512)		Rats (hexamethonium)	
(520)		Rats (hexamethonium)	
(306)		Rats (hexamethonium)	
(120)	Rabbits (coronary effluent)		
(121)		Rabbits (renal nerve section)	
(525)			Rats (capsaicin, CGRP levels in plasma)
(117)	Rabbits (coronary effluent)		
(423)		Rats (hexamethonium)	
(118)	Rabbits (coronary effluent)		
(119)	Rabbits (whole blood)		
(471)			Rabbits (capsaicin, CGRP levels in plasma)
(161)		Rats (hexamethonium)	

Table 1.3 Summary of studies on the mechanisms of transfer of protection by RIPC. For each study, the animal type and agents/intervention used to demonstrate the role of various factors *(in brackets)* are reported. In all studies pharmacological agents/interventions were administered in advance of the RIPC stimulus. Negative studies are in red.

1.5 RIPC in humans; scope of this thesis

The mechanism of RIPC has yet to be investigated in humans in vivo and this is the principal aim of the experimental work described in this thesis. To study the mechanism of RIPC in humans, it is essential to induce transient quantifiable ischaemia-reperfusion injury *in vivo* without risk of harm. Our group recently developed a model that satisfies these criteria in humans (243;244). In these studies of healthy volunteers, the arm was made ischaemic for 20 minutes after which reperfusion occurs. This caused transient dysfunction of the vascular endothelium of resistance and conduit vessels in the arm (measured by the degree of vasodilatation in response to endothelium-dependent stimuli). In subsequent studies from our laboratory, RIPC was initiated by three five-minute cycles of ischaemia to the contra-lateral arm, and largely prevented endothelial dysfunction of resistance vessels in response to IR *(figure 1.21)* (243). Although the above observations do not answer the ultimate question whether RIPC can be protective to the human myocardium, they demonstrate that the phenomenon exists in humans.

Using this model, in *chapter 3* I have undertaken an investigation of the relationship between differing RIPC stimuli and protection against endothelial IR injury of conduit vessels in healthy volunteers. One small clinical study of RIPC (8 patients in total) used an RIPC stimulus of 2 cycles of 3 minutes of arm ischaemia applied 10 minutes before global cardiac ischaemia (181). No effect to reduce ischaemic injury was demonstrated although there was evidence of enhanced anaerobic glycolysis. It is possible that the RIPC stimulus used in this study was of insufficient strength to cross the preconditioning threshold and induce protection.

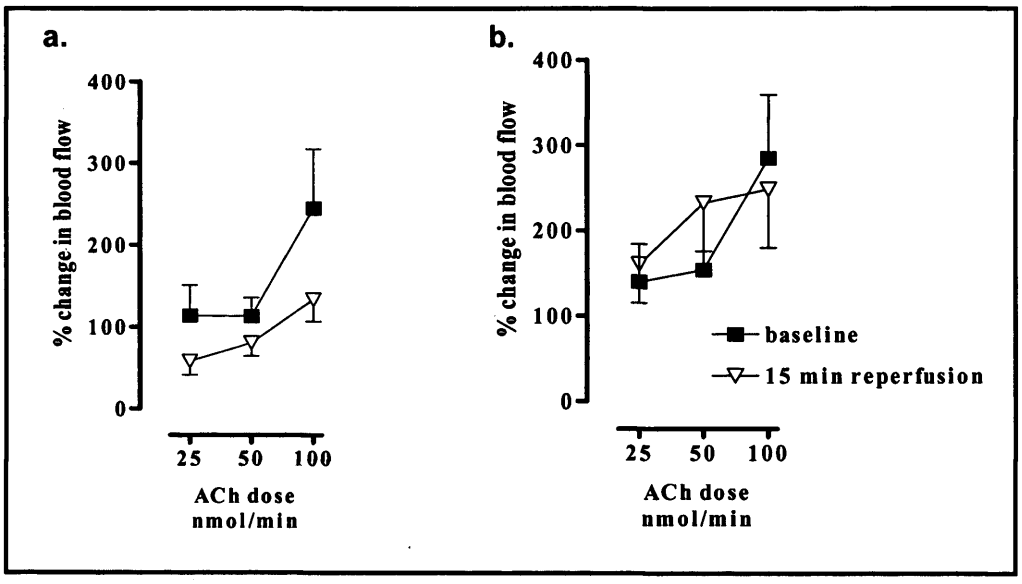


Figure 1.21 Original data demonstrating resistance vessel endothelial dysfunction caused by ischaemia-reperfusion of the arm (a) and how this is prevented by remote ischaemic preconditioning (RIPC) (b) (Kharbanda *et al*, Circulation; 106: 2881, 2002).

In *chapter 4* I have examined the temporal features of RIPC, to assess whether there are two phases of protection in humans and if protection can be sustained by repeated application of a RIPC stimulus. Late effects of RIPC have been demonstrated recently in humans, with evidence for suppression of pro-inflammatory genes in circulating leucocytes 24 hours after 3 cycles of arm ischaemia (258). *Chapter 5* investigates the role of the autonomic nervous system in the spread of RIPC, by performing studies in subjects treated with an autonomic ganglion blocking drug. *Chapter 6* determines the role of K_{ATP} channels in RIPC using the blocker glibenclamide. In *chapter 7*, I have investigated whether the effect of RIPC to diminish endothelial injury occurs during the ischaemic or reperfusion phase of IR injury. *Chapter 8* investigates ischaemia-reperfusion injury in paediatric renal transplantation and whether RIPC has any protective effects against clinical IR in this setting.

Chapter 2:

General Methods

2.1 Assessment of endothelial function in humans *in vivo*

Several techniques may be used to assess arterial endothelial function in vivo ranging from the biochemical to the physiological. No single measure describes all aspects of its varied function. Furthermore, endothelial function is not uniform between different vascular beds or at different sites in the same vascular bed. Endothelial function in veins may differ from that in arteries, resistance arteries may respond differently to larger conduit arteries and the endothelium at arterial branch points exposed to turbulent flow may behave differently from that in areas exposed only to laminar flow (344). The role of the endothelium in the regulation of vascular tone has become one of the most comprehensively studied areas of vascular biology. A variety of biochemical assays [von Willebrand factor (vWF), platelet activating factor inhibitor type 1 (PAI-1), tissue plasminogen activator (tPA), and thrombomodulin] and physiological assays [flow-mediated dilation (FMD) or receptor mediated vasodilation (venous occlusion plethysmography)] have been used to assess endothelial function. The correlation between these biochemical and physiological measures of endothelial dysfunction is weak in the clinical setting (548). In part, this reflects the lack of tissue and cell specificity of these biochemical substances.

Physiological measures of endothelial function remain central to the assessment of the endothelium. Such techniques rely on the measurement of blood vessel tone or diameter in response to endothelium-dependent stimuli that cause vasodilation or vasoconstriction. In the studies described in this thesis, I have chosen to examine dilation of conduit arteries in response to a high-flow stimulus (flow-mediated dilation; FMD). This technique was chosen because it is reproducible, reliable, and non-invasive. In addition, the mechanisms of FMD have been well characterised and

under defined conditions reflect the activity of the NO pathway *(see section 2.1.1.1)* (230;344). Moreover, forearm conduit artery endothelial function has been shown to correlate well with endothelial function in the coronary vasculature (17). Throughout these studies, I have performed parallel assessment of intrinsic smooth muscle responsiveness. The smooth muscle response is the final common pathway both for the dilator signal derived from the endothelium and for exogenous endothelium independent vasodilators.

2.1.1 Flow-mediated dilation

Studies performed in the 1980s indicated that large arteries (conduit vessels) responded to alterations in blood flow by increasing vessel diameter (16), and that this phenomenon was endothelium-dependent in vitro (396). Early in vivo techniques were aimed at the assessment of coronary artery endothelial function, and were necessarily invasive and difficult to repeat over time. In 1989, a non-invasive ultrasound technique was developed to assess endothelial function in peripheral conduit vessels (16). This technique is based on the observation that conduit vessels respond to an increase in flow (or more precisely shear stress) by dilating (96). This phenomenon is designated *flow-mediated dilation (FMD)* and has subsequently been shown to correlate with invasive testing of endothelial function in the coronary circulation (17) and more importantly, with cardiovascular outcome (355). FMD provides a dynamic assessment of conduit artery endothelial function that is both accurate and reproducible (451). The majority of studies to date have assessed FMD in the brachial artery (60), but FMD has also been studied in the radial (101) and superficial femoral arteries (107). The dilation of human conduit arteries in response to increased flow has been shown to be mediated in part by endogenous NO, and can

be reduced by the inhibition of NO synthesis (230;344). FMD of the brachial artery has been used to study endothelial dilator function in a variety of cardiovascular risk groups and has been used in large clinical studies as a "surrogate" marker of cardiovascular risk (84). Impaired endothelial function, assessed in this manner, has not only been reported in individuals with symptomatic atherosclerotic disease (87;269), but also in asymptomatic children and adults with elevated cardiovascular risk factors such as smoking (82;83), hypercholesterolaemia (439;477), hypertension (129;160), diabetes mellitus (123;476) and hyperhomocysteinaemia (39;522). The non-invasive nature of FMD allows studies to be conducted among children, adolescents and young adults, which may greatly enhance our insight into the causes, development, and pathophysiological mechanisms of cardiovascular disease. In studies in this thesis, I have used FMD to assess the effects of IR on endothelial function of the brachial artery in the arm of healthy volunteers.

2.1.1.1 Physiology of FMD

FMD relies on the observation that blood vessels dilate in response to high blood flow. Increased conduit artery blood flow is generated after transient forearm ischaemia, which induces reactive hyperaemia on cuff release. The increase in arterial diameter in response to this high flow stimulus is measured and the magnitude of this dilation is taken as a measure of the activity of the NO pathway. The precise mechanism for the acute detection of the hyperaemia-induced increase in shear stress and the subsequent signal transduction to modulate vasomotor tone is not fully understood. It has been suggested that vascular endothelial cell hyperpolarisation, due to shear stress-induced K^+ channel opening, results in Ca^{2+} entry to the cytoplasm (94;367) and activation of endothelial nitric oxide synthase (eNOS, NOS3) (97). The

subsequent generation of NO appears to account for FMD (230). It has been demonstrated that endothelial denudation in vitro or administration of NOS inhibitors in vivo abolishes FMD in a variety of arterial vessels (96). In humans it is unknown whether other mediators, such as prostaglandins or the putative endothelium-derived hyperpolarising factor (EDHF) mediate FMD (459)

2.1.1.2 Experimental technique

All FMD studies in this thesis were performed at the Vascular Physiology Unit, Department of Cardiology, Institute of Child Health, University College London.

2.1.1.2.1 Subject preparation

Flow-mediated vascular reactivity is affected by numerous factors including temperature, food, medication and sympathetic stimuli (96). According to the guidelines of the International Brachial Artery Reactivity Task Force (96), to minimise the effect of these confounding factors on FMD measurements, subjects should fast for at least 4 hours before the study, and they should be studied in a quiet, temperature-controlled room. All vaso-active medication should be withheld for at least 4 half-lives. In addition subjects should not exercise and should not ingest substances that might affect FMD (caffeine, high-fat foods), or use tobacco for at least 4 to 6 hours before the study. In studies presented in this thesis, the above guidelines were followed, and healthy subject exclusion criteria were age>45 years, smoking, use of vaso-active medication and pregnancy.

2.1.1.2.2 Image acquisition

The subject is positioned supine with the arm in a comfortable position. High-resolution vascular ultrasound is used to image the brachial artery in longitudinal section. The operating parameters of the ultrasound machine are adjusted to maximise the differentiation between the arterial lumen and wall. Once a stable clear image is obtained *(figure 2.1)*, the transducer position is fixed over the artery using a stereotactic clamp and micrometer adjustment screws *(figure 2.2)*, which permit adjustment in the coronal and sagittal plane to maintain a constant, focused image of the vessel being studied. In this thesis, studies were performed using a standard Acuson XP ultrasound system (Acuson, Mountain View, California, USA) with a 7MHz linear array transducer.

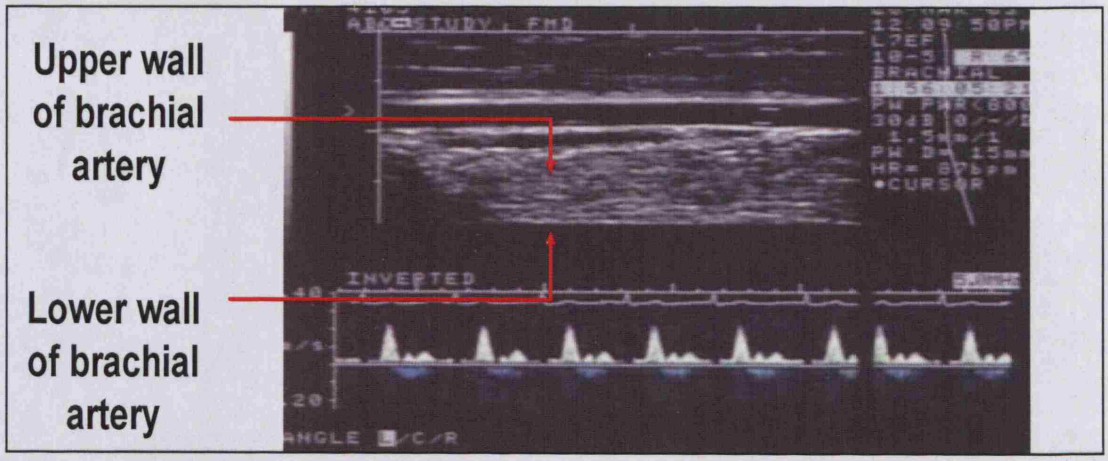


Figure 2.1 Stable image of a longitudinal section of the brachial artery.

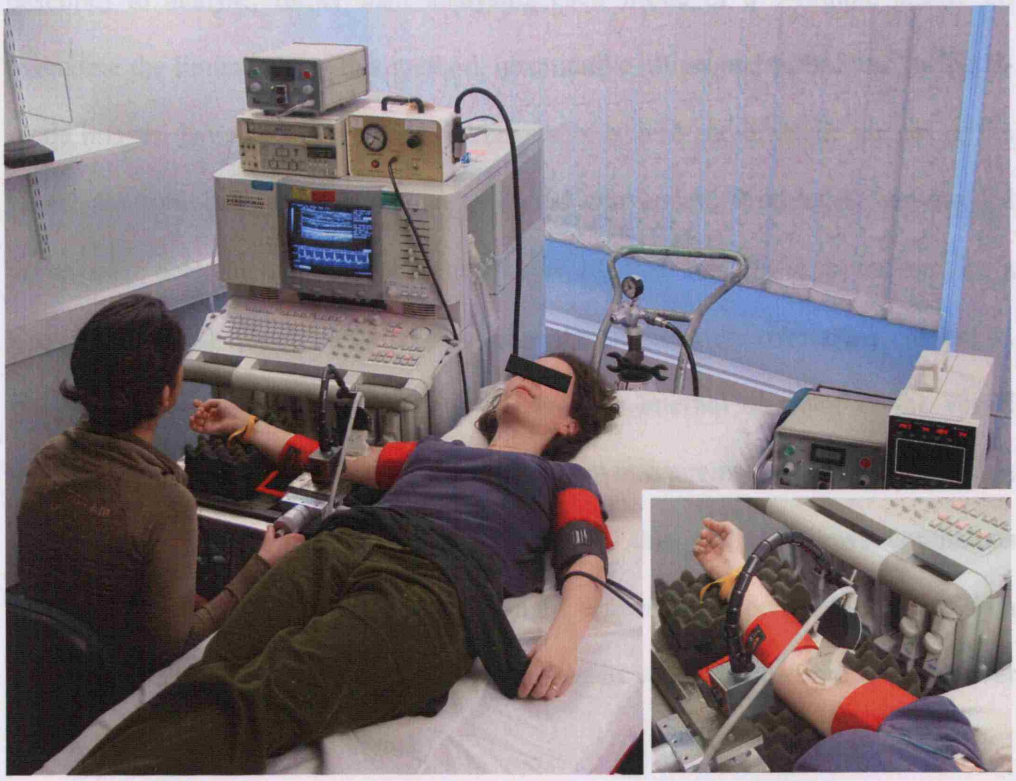


Figure 2.2 The experimental set up used to record flow-mediated dilation demonstrating stereotactic clamp (inset) and micrometer adjustment screws.

2.1.1.2.3 Measurement of arterial diameter

Several methods are available to measure arterial diameter, and these have been developed since the first description of the technique in 1989 (84). In the original description of the technique, the image of the vessel was recorded in M-mode. Arterial diameter was measured using manual on-screen callipers, which were placed at the M-lines of the anterior and posterior walls, at a single point in the artery. This method is extremely labour intensive. In order to optimise analysis time, researchers have identified specific time points of interest (baseline, peak dilation and recovery of

baseline) to analyse, rather than analysing each frame of a 9-minute study. To overcome the limitations of this method, quantitative ultrasound techniques, using B-mode images, have been developed. Ultrasound acquired end-diastolic images of the vessel are subject to 8 bit analogue to digital conversion. Proprietary commercial digital edge detection software (Brachial Tools, Medical Imaging Applications, Iowa City, Iowa, USA) calculates the internal diameter of the artery over a 2-3 cm length. Images are acquired at 3-second intervals and the internal diameter of the vessel measured for each image *(figure 2.3)*. This allows measurement of a longer segment of the artery than previous methods. Moreover, this method allows continuous assessment of vessel diameter throughout an experimental protocol, which results in a more comprehensive description of the changes in vessel diameter that occur over time in response to a flow stimulus.

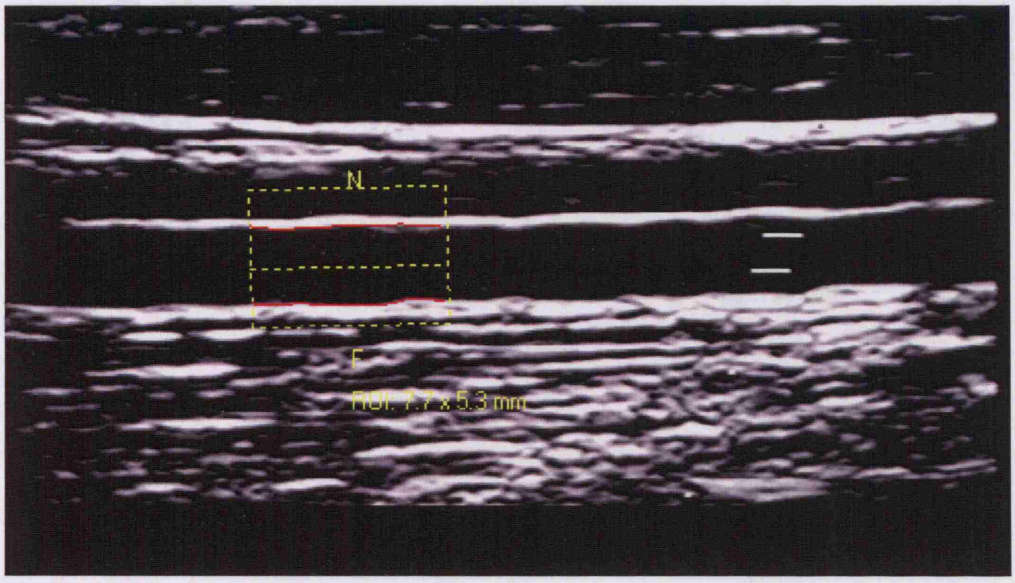


Figure 2.3 Region of interest box during automatic diameter measure.

2.1.1.2.4 Data analysis and presentation of arterial diameter

Comparison of the arterial dilator response in a single subject measured on multiple occasions, or between groups of subjects is possible if there are no major differences in basal blood flow or arterial diameter. Data can be expressed as absolute dilation, or as percentage change from baseline. It is possible to plot the whole time course of the dilation together with return to baseline and then calculate both the peak dilation and the area under the curve for the whole dilation profile *(Figure 2.4)*. In studies in this thesis, diameter changes induced by an increased flow stimulus are expressed as peak percentage change from baseline.

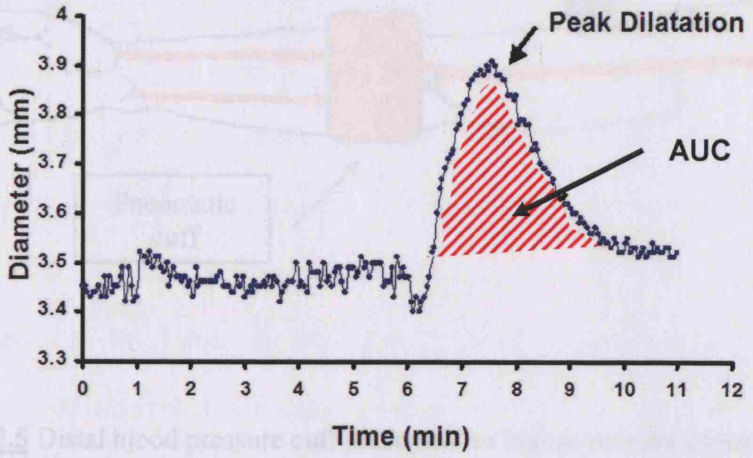


Figure 2.4 Area under the curve (AUC) of diameter / time profile.

2.1.1.2.5 Inducing high blood flow in a conduit artery

In order to determine FMD in the brachial artery, high blood flow is generated by inducing reactive hyperaemia. A blood pressure cuff is positioned 1-2 cm below the antecubital fossa, *distal* to the site of brachial artery diameter recording *(Figure 2.5)*. The blood pressure cuff is inflated to supra-systolic pressure, causing forearm ischaemia, for five minutes (290). At cuff release there is a rapid, transient increase in blood flow which lasts approximately 90 seconds, with peak flow observed within 30 seconds following cuff release *(figure 2.6)*.

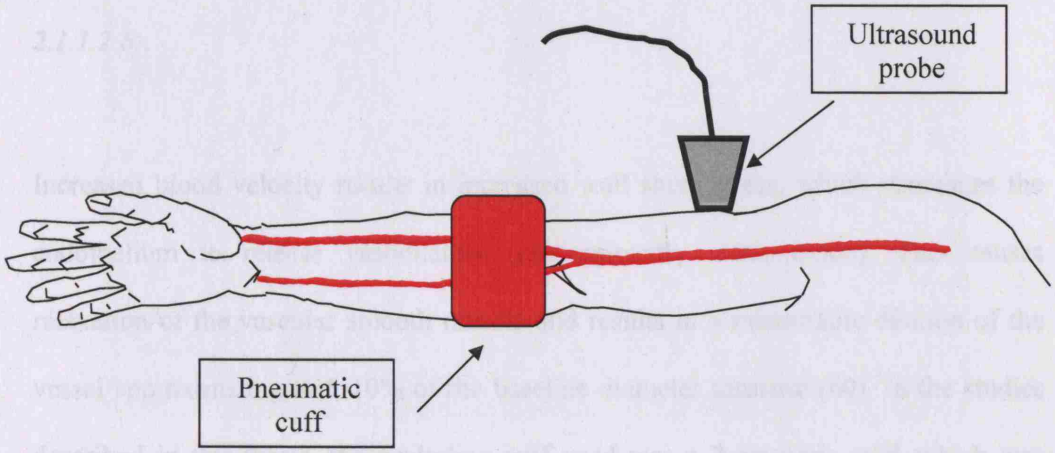


Figure 2.5 Distal blood pressure cuff placement to induce reactive hyperaemia.

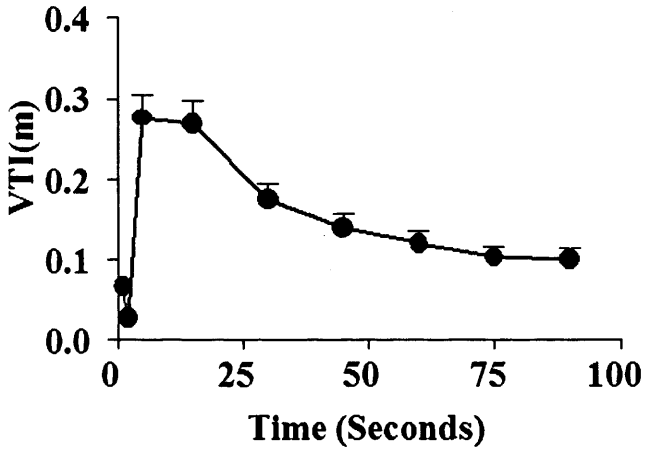


Figure 2.6 Blood flow / time profile. *VTI: velocity-time integral (see section 2.1.1.2.6).*

Increased blood velocity results in increased wall shear stress, which stimulates the endothelium to release vasodilators (predominantly nitric oxide). This causes relaxation of the vascular smooth muscle and results in a measurable dilation of the vessel approximating to 5-10% of the baseline diameter measure (60). In the studies described in this thesis, the occluding cuff used was a 7-cm wide cuff, which was placed *distal* to the segment of artery being studied. Other investigators have placed the cuff *proximal* to the study segment, which results in a greater dilator response, but this dilation is thought to result from a combination of increased flow and ischaemia of the conduit vessel. The latter is a complex signal which is mediated by the release of local factors in addition to NO (46).

2.1.1.2.6 Measurement of blood flow

Blood flow velocity was measured using pulsed wave Doppler. The Doppler signal is the velocity-time profile for a single cardiac cycle and is displayed as a spectral Doppler curve. The area under the curve of the velocity-time profile is the velocity-time integral (VTI) *(figure 2.7)*, and approximates to the average distance, measured in metres, travelled by a pulse of blood during one cardiac cycle, typically at rest 0.01-0.05 m.

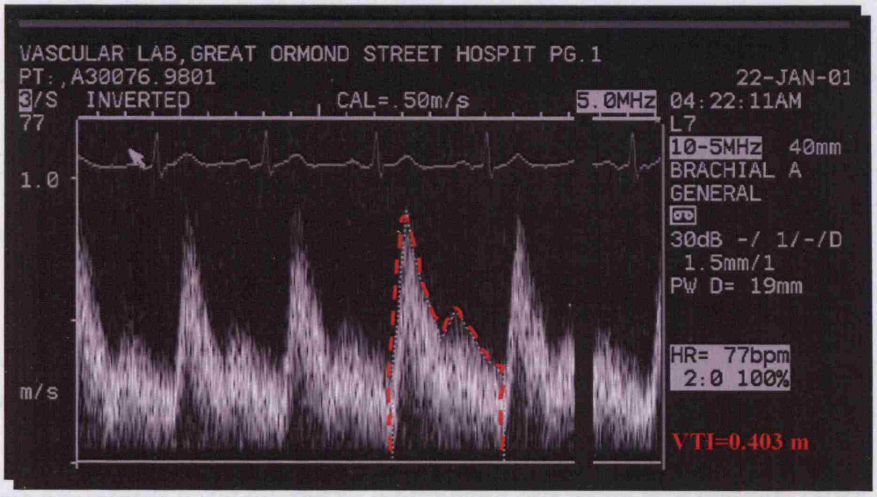


Figure 2.7 Calculating the velocity time integral.

VTI = Velocity (m/s) x time (seconds)

VTI = [Distance (m) / time (seconds)] x time (seconds)

VTI = Distance (m)

Volume flow is that volume of blood that passes a point in a specified period. Assuming that we can approximate a section of artery to a cylinder, we can calculate the blood flow volume as follows:

Volume / cardiac cycle = artery cross-sectional area x average distance travelled by pulse $= \pi r(t)^2 \int v(t)\, dt$,

where $r(t)$ ***= the measured instantaneous vessel radius,***

and $v(t)$ ***= the instantaneous blood velocity.***

The Doppler signal is measured at an angle of approximately 70° to the axis of the blood vessel, giving $\int v(t)\, dt = \cos 70° \times$ measured VTI, and thus:

Volume / cardiac cycle $= \cos 70° . \pi r(t)^2 . VTI$

Volume per minute is calculated by multiplying this value by heart rate (HR).

Volume / min $= HR \cos 70° . \pi r(t)^2 . VTI$.

VTI can be considered proportional to volume flow per minute if heart rate, the Doppler angle of incidence and arterial diameter (2 x arterial radius) remain constant during the period of study. Measurement of the radius for the period of peak flow (up to 30 seconds after cuff release) shows that $\Delta r <<$ (tends to zero, compared with) baseline radius, i.e. there is no significant change in arterial radius (and diameter) during the period of peak flow *(figure 2.8)*. In addition during the same time period heart rate and Doppler angle are also constant, and thus it can be concluded that peak

volume flow following cuff release coincides with peak VTI. *Figure 2.8* below shows both VTI and the arterial dilator response (equal to Δr/r) as a function of time.

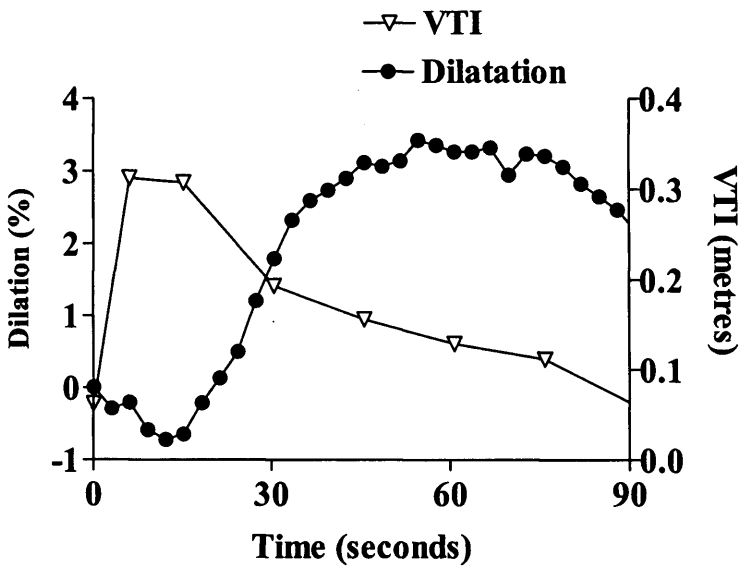


Figure 2.8 VTI & dilation as a function of time.

The most significant error in the assumption that the volume flow per minute is proportional to VTI is due to variation in the radius. To calculate this error, the square of the radius at time t, $r(t)^2$, was expanded as follows:

$$r(t) = r(t=0) + \Delta r$$

$$r^2(t) = (r(t=0) + \Delta r)^2$$

$$r^2(t) = r^2(t=0) + 2r(t=0)\Delta r + \Delta r^2$$

$$= r^2(t=0)[1+2\Delta r/\, r(t=0) + \Delta r^2/\, r^2(t=0)]$$

In the above formulae, r(t=0) is the arterial radius at the time when the blood pressure cuff is released and it is equal to the radius of the artery prior to forearm ischaemia

[baseline; r(baseline)]. Now as $\Delta r << r(t=0)$, we can ignore the last term, and the error in volume is:

$$Volume / min = HR(t=0) \cos 70^{o}.\pi r(t=0)^{2}.VTI\,[1 + 2\Delta r / r(t=0)]$$

So fractional error is typically $(2\Delta r / r(t=0))$, and thus varies with dilation. From *figure 2.8*, the maximum error occurs at $t \approx 60s$ (time of maximum dilation and thus Δr) and is typically ≈6%. For the majority of the period of interest (30 seconds following cuff release), however, this is negligible, as arterial radius remains almost constant and approximately equal to r(t=0). Consequently the following formula can be used to calculate peak volume follow per minute during reactive hyperaemia:

$$Volume / min\ (peak) = HR(at\ time\ of\ peak\ VTI) \cos 70^{o}.\pi r(t=0)^{2}.VTI(peak)$$

2.1.1.2.7 Data analysis and presentation of arterial flow

Blood flow is the stimulus to endothelium-dependent arterial dilation and can be expressed in several ways: absolute volume flow, ratio of peak to baseline absolute volume flow, percentage change in volume flow between baseline and peak, or as area under the curve (AUC) for the whole flow profile as it returns to baseline values over a period of 90 seconds. These parameters can similarly be expressed for the VTI. In studies in this thesis, baseline and peak VTI were calculated and the ratio of peak to baseline absolute volume flow per minute was determined using the following formula:

$$\frac{Volume\ /\ min(peak)}{Volume\ /\ min(baseline)} = \frac{HR(at\ time\ of\ peak\ VTI)\ \cos 70^{\circ}.\pi r(t=0)^2.VTI(peak)}{HR(baseline)\ \cos 70^{\circ}.\pi r(baseline)^2.VTI(baseline)}$$

Given that r(t=0) is approximately equal to r(baseline) the above formula can be written as follows:

$$\frac{Volume\ /\ min(peak)}{Volume\ /\ min(baseline)} = \frac{HR(at\ time\ of\ peak\ VTI).VTI(peak)}{HR(baseline).VTI(baseline)}$$

In most studies in this thesis no significant changes in heart rate were observed *(see result chapters)*. As it could be assumed that heart rate values at baseline and at the time of peak VTI would be similar, the peak to baseline ratio of VTI could be considered an accurate representation of the peak (hyperaemia) to baseline ratio of flow volume. However, to avoid the confounding effect of small changes in heart rate and the risk of error in the measurement of the flow stimulus in this thesis, heart rate values were recorded at the same time as VTI measurements at baseline and peak hyperaemia and were used in the calculation of the volume flow per minute ratio.

2.1.1.2.8 Experimental protocol

Longitudinal, ECG-gated end-diastolic images were acquired every 3 seconds, using customized software, and arterial diameter over a 1-2 cm segment was determined for each image. Pulsed wave Doppler was used to measure blood flow velocity expressed as the velocity time integral (VTI) for a single cardiac cycle. Brachial artery diameter and VTI were measured for 1 minute (baseline), during 5 minutes of reduced blood flow (induced by inflation of a pneumatic cuff to *300 mmHg* placed at the forearm, immediately below the antecubital fossa, *distal* to the segment of brachial artery being analysed), and for 5 minutes during reactive-hyperaemia after release of the blood pressure cuff. The blood flow stimulus was expressed as the ratio of peak to baseline volume flow per minute. Dilation was expressed as peak percentage dilation.

2.1.1.3 Assessing intrinsic smooth muscle function; endothelium-independent dilation

To assess intrinsic smooth muscle reactivity (endothelium independent dilation), glyceryl-trinitrate (GTN) is given sublingually and arterial dilation is recorded. GTN supplies NO directly to vascular smooth muscle and cause relaxation, which is independent of the endothelial cell layer. Early in the development of this technique, conventional anti-anginal doses of GTN (400μg) were given (290). This had two major disadvantages; firstly, administration was commonly associated with headache, which though short lived and self-limiting, is undesirable. Secondly, the dilator response of the conduit vessel to large doses of GTN was far in excess of that following reactive hyperaemia. This meant that it was possible to miss subtle differences in intrinsic smooth muscle function because of the excess of NO applied to the system. Cross *et al* (100) demonstrated that a smaller dose of GTN (25μg) can

induce a conduit vessel dilator response of similar magnitude to the one induced by a flow stimulus in a cohort of chronic renal failure patients. Subsequent work by Kharbanda *et al* (244) confirmed these observations in healthy volunteers. In the latter studies peak vasodilation in response to GTN occurs 3-4 minutes following GTN administration.

In studies in this thesis, a 25μg GTN dose was administered sublingually to test the intrinsic function of the vascular smooth muscle in the brachial artery. Brachial artery diameter was measured, as described above, for 1 minute, after which GTN was administered. Brachial artery diameter was measured for another five minutes following GTN administration. As for FMD, dilation in response to GTN was expressed as peak percentage dilation.

To ensure that the 25μg dose of GTN induced the same degree of brachial artery dilation as increased flow, I compared FMD and GTN-induced dilation in a group of 7 healthy volunteers. As it can be seen in *figure 2.9*, the dilator response of the brachial artery of 25μg of sublingual GTN (9.3±2.0%) matched FMD values (9.2±1.7%) in this group (p=0.9813, paired student's *t*- test).

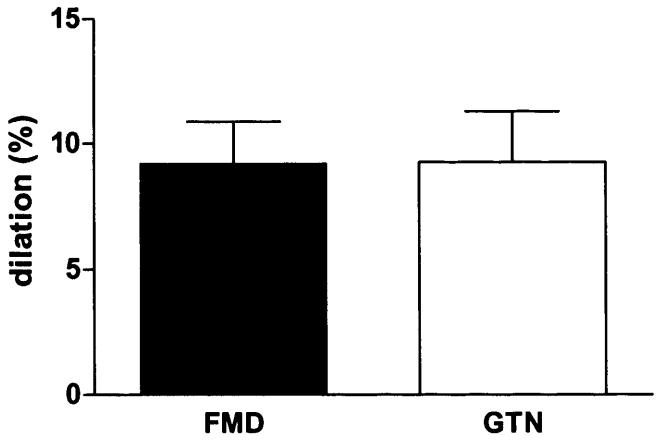


Figure 2.9 FMD and sublingual GTN-induced dilation in the brachial artery of healthy volunteers (n=7).

2.1.1.4 Accuracy and reproducibility of the technique

In any measuring technique, there are a number of potential sources of error. Ideally, a measure should be accurate and reproducible over both time and between observers. The problem of measurement inaccuracy in FMD was addressed by Sorensen *et al.* (451) using high resolution ultrasound to record the absolute dimensions of 'phantom' arteries of known diameter (mock blood vessels with the same ultrasound characteristics as the original embedded in a block of latex). These investigators recorded the observer's ability to distinguish between small differences in phantom vessel diameters. Different observers were able to discern the absolute diameter correctly to within 0.04 mm and were able to distinguish between pairs of phantom arteries with diameter differences 0.1 mm in 61% of cases using this technique (451). These data suggest that the technique is accurate.

In addition, it is also important to establish reproducibility of this technique over time (intra-observer variation) and between observers (inter-observer variation) since, in the studies described in this thesis, individuals have been examined on multiple occasions. In order to reliably detect a treatment effect, we need to be confident that we are able to distinguish between changes in the measure that relate to background measurement variability (noise) and changes in the signal that relate to the intervention. Sorensen *et al.* have assessed inter-observer variability using the technique of FMD in a study, in which four independent observers recorded arterial diameter (451). They reported that no measurement was more than 0.1 mm from the measure made by the other 3 observers and the coefficient of variation was 1.8% (451). In studies presented in this thesis, two independent observers assessed a random selection of traces from 20 healthy volunteers. The results for two observers are shown in *figures 2.10* and *2.11*.

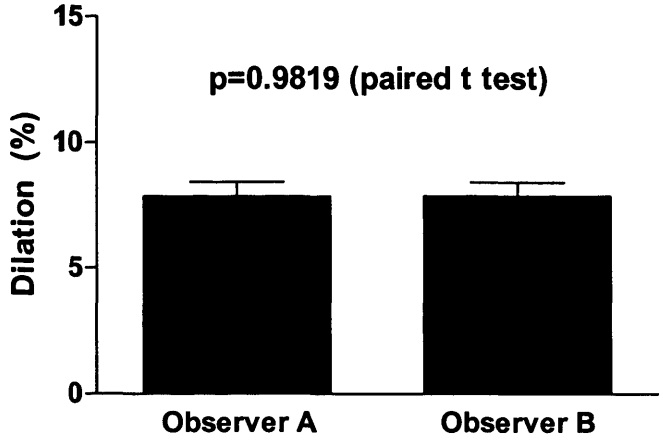


Figure 2.10 Inter-observer variability in the measurement of FMD.

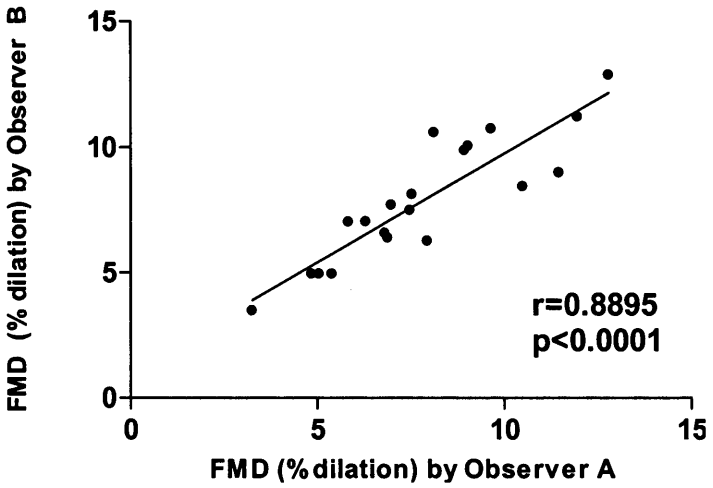


Figure 2.11 Correlation between brachial artery FMD measurements performed by 2 independent observers.

In additional unpublished work carried out in the Vascular Physiology Unit by Mrs Ann Donald, the variation of FMD over time has been investigated using this technique. 36 healthy volunteers were examined repeatedly by the same observer over a period of three months. FMD was measured at baseline and then sequentially one week, and one month later and there was good agreement in FMD values as shown by Bland-Altman plots (*figure 2.12*). The coeeficient of variation for FMD measurement performed one week and one month apart were 9.95% and 6.05% respectively.

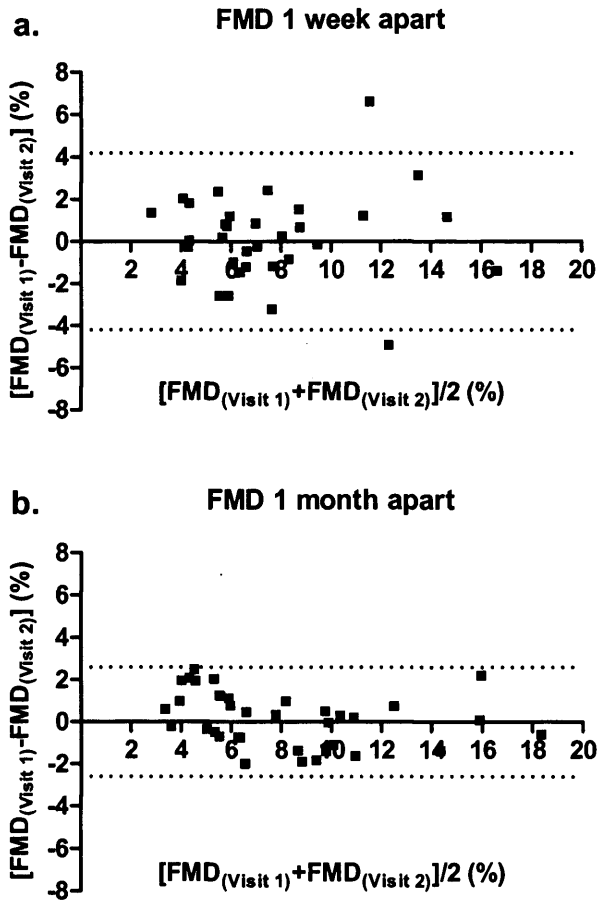


Figure 2.12 Intra-observer variability in the measurement of FMD. Bland-Altman plots for FMD measurements performed at baseline and then one week (a) or one month (b) later. The FMD differences between visits [$FMD_{(Visit\ 1)}$–$FMD_{(Visit\ 2)}$] were plotted against the mean FMD value for these visits [$FMD_{(Visit\ 1)}$+$FMD_{(Visit\ 2)}$]/2. Dotted lines represent 2 x SD of the differences in FMD between visits.

Any measure that requires observer judgement in the processing of data before generating a result is prone to bias. In this thesis, observer bias is minimised by the use of wholly automated computerised analysis packages. In some studies manual estimates of arterial diameter were made to ensure vessel diameters had returned to baseline. However these measurements did not influence the protocol so the risk that this would introduce bias is small.

2.2 Biochemical assays

All biochemical assays in this thesis were performed at the Wellchild Laboratory, Guys Hospital, London, under the supervision of Dr. R.N. Dalton and Dr. C. Turner. These analyses were performed as part of a clinical study of the effect of RIPC on renal IR injury during transplantation (*chapter 8*).

2.2.1 Measurement of urinary retinol binding protein by enzyme-linked immunosorbent assay (ELISA)

2.2.1.1 Retinol binding protein

Low molecular weight (LMW) proteins have a molecular weight below 50,000 Daltons (Da) and include β_2-microglobulin, lysozyme and retinol-binding protein (RBP). These proteins have a relatively high glomerular sieving coefficient and are subject to extensive reabsorption by the renal tubules (mainly in the proximal tubule).

Retinol-binding protein (RBP) is the carrier protein for vitamin A in human plasma (240). The RBP molecule consists of a single polypeptide chain of 182 amino acids and has a molecular weight of approximately 21,000Da (240). The molecular confirmation of RBP is almost spherical *(figure 2.13)*. RBP is synthesised and secreted by the liver, and 95% of its degradation occurs via the kidney (491).

In plasma most RBP is bound to pre-albumin (240), a plasma protein with four identical subunits, each containing a binding site for RBP (387). The unbound (free) form of plasma RBP accounts for 10-15% of the total RBP (387), and its mean concentration has been found to be between 4.1-8.7mg/l in healthy adults (42) and

15-47mg/l in healthy children (125). Unbound plasma RBP is freely filtered by the glomerulus and is 99.9% reabsorbed by the proximal tubule (481). RBP is reabsorbed from the urine by a process of endocytosis, which occurs at the luminal border of the renal tubular epithelium (61;327) and is highly energy-dependent (320). Thus, RBP is almost fully recovered by the kidney, and very small amounts are found in the urine of healthy individuals (481).

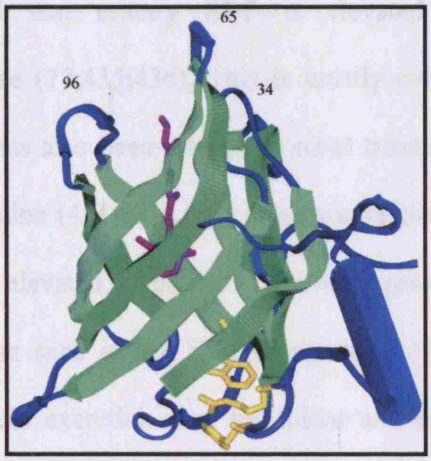


Figure 2.13 Retinol-binding protein.

The daily urinary excretion of RBP ranges between 40µg/24 hours and 220µg/24 hours with a mean value of 110µg/24 hours in normal adult subjects (388). The mean urinary RBP concentration has been determined by Topping *et al* at 64µg/l (483). The urinary RBP concentration has more frequently been expressed as the ratio to urine creatinine excretion and studies have reported mean values that range from 5.8 to 11.4µg/mmol of creatinine for healthy adults (36;43;316;483). In children, the urinary excretion of RBP is high at birth compared to adult values, but declines over the first six months of life, and beyond two years, values are close to adult norms (mean 14.5µg/mmol of creatinine; range 3.8 to 60 µg/mmol of creatinine) (450).

The handling of RBP by the kidney is independent of glomerular function (481). Any increase in urinary excretion reflects a *decrease in tubular reabsorption*, and changes in glomerular permeability do not result in increased filtration. Due to this RBP has been evaluated as possible indicator of renal tubular dysfunction. Butler and Flyn (74) were the first to demonstrate that in patients with proximal tubular disease, urine contains proteins, the majority of which have a low molecular weight. Since then it has been established that urinary RBP is elevated in patients with renal tubulointerstitial disease (77;435;436). This is mostly evident in the renal Fanconi syndrome (65). RBP has also been studied in renal transplantation, primarily in the detection of graft rejection (481;521). The urinary excretion of RBP and other LMW proteins is frequently elevated in patients following renal transplantation, often to levels resembling those seen in the Fanconi syndrome (403). There is a baseline increase in RBP protein excretion post transplant and episodes of acute rejection cause a further increase in excretion (335).

In the clinical study presented in *chapter 8* in this thesis, urinary RBP was evaluated as a marker of IR injury to the kidney in children undergoing renal transplantation. RBP was chosen due to its greater stability in urine compared to other LMW proteins (481). Urinary pH is an important factor in determining the stability of LMW, and it has been shown that RBP is stable in urine samples at any physiological pH (124).

Several analytical methods have been developed for the measurement of RBP in urine, that include radioimmunoassays (36), latex immunoassays (43) and enzyme-linked immunosorbent assays (ELISA) (483). The technique of ELISA is safe, inexpensive and technically straightforward. As a result, it has been used in the

majority of studies to date for the measurement of urinary RBP. The ELISA technique for RBP measurement used in this thesis was developed by Tomlinson *et al* (482) and was based on a common two site immunoenzymometric *"double-sandwich"* procedure.

2.2.1.2 Rationale of "double-sandwich" ELISA

ELISA's combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies coupled to an easily assayed enzyme that possesses a high turnover number. One of the most useful of these immunoassays is the two antibody "double-sandwich" ELISA, which is used to measure the concentration of a specific compound in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, it can be used to determine the absolute amount of antigen in an unknown sample. The "double-sandwich" ELISA requires two antibodies which bind epitopes on the antigen (compound measured) that do not overlap. This can be accomplished with either two monoclonal antibodies that recognise discreet sites on the antigen or one batch of affinity-purified polyclonal antibodies.

To utilize this assay, one antibody (the *"capture"* or *first* antibody) is purified and bound to a solid phase typically attached to the bottom of a plate well. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a conjugated *second* antibody (the *"detection"* antibody) is allowed to bind to the antigen, thus completing the "sandwich". The assay is then quantitated by measuring the amount of enzyme-labelled secondary antibody bound to the matrix, through the use of a colorimetric substrate. Unlike Western blots, which

use precipitating substrates, ELISA procedures utilize substrates that produce soluble products. Ideally the enzyme substrates should be stable, safe and inexpensive. Substrates are colourless and are converted by the enzyme, coupled to the secondary antibody, to a coloured product, e.g., p-nitrophenylphosphate (pNPP), which is converted to the yellow p-nitrophenol by alkaline phosphatase. Substrates used with horse-radish peroxidase (HRP) include 2,2'-azo-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), o-phenylene diamine (OPD) and 3,3'5,5'- tetramethylbenzidine base (TMB), which yield green, orange and blue colors, respectively.

Figure 2.14 summarises the basic principles of the "double-sandwich" ELISA.

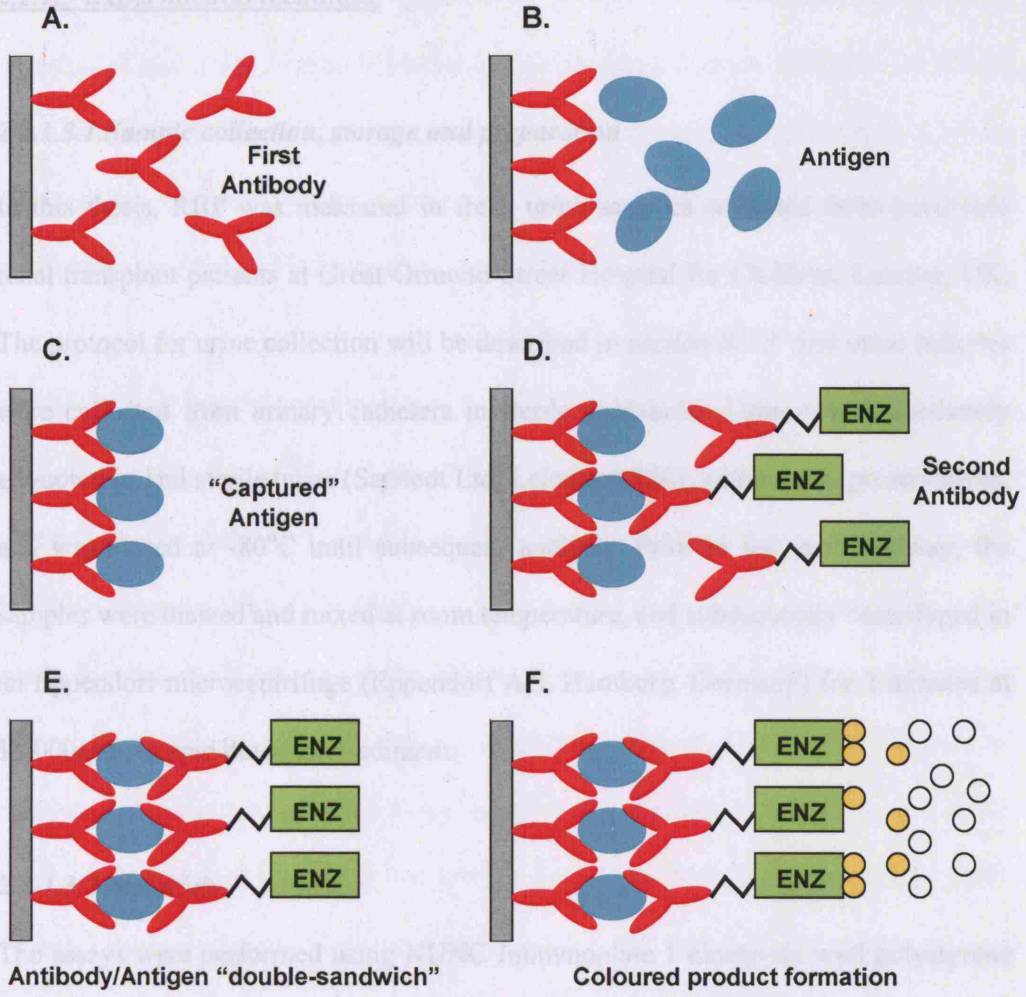


Figure 2.14 Basic principles of "double-sandwich" ELISA: (A). First antibody is added and allowed to passively attach to a plate well surface (solid phase) by incubation in buffer; (B). Unattached antibodies are washed away and antigen (compound to be measured in unknown sample) is added; (C). Antigen is "captured" by coating antibodies during incubation. Unbound antigen is then washed away; (D). Conjugated second antibodies directed against the antigen are added; (E). Binding of the second antibody during incubation completes the "double-sandwich". Free second antibody is washed away; (F). Colourless substrate is added and is converted to coloured product by the antibody-bound enzyme. The colour is then quantified in a spectrophotometer.

2.2.1.3 Experimental technique

2.2.1.3.1 Sample collection, storage and preparation

In this thesis, RBP was measured in fresh urine samples collected from paediatric renal transplant patients at Great Ormond Street Hospital for Children, London, UK. The protocol for urine collection will be described in *section 8.2.3*. 5ml urine samples were collected from urinary catheters in sterile containers. Urine was immediately aliquoted in 1ml sterile tubes (Sarstedt Ltd, Leicester, UK), without any preservatives, and was stored at -80°C until subsequent analysis. Prior to the immunoassay, the samples were thawed and mixed at room temperature, and subsequently centrifuged in an Eppendorf microcentrifuge (Eppendorf AG, Hamburg, Germany) for 2 minutes at 16,000rpm, to spin down any sediment.

2.2.1.3.2 Materials

The assays were performed using NUNC Immunoplate 1 ninety-six well polystyrene microtitre plates, purchased from Gibco (Invitrogen-Gibco Ltd., Paisley, UK). Antibodies were polyclonal rabbit antibodies raised against human RBP. An unconjugated antibody preparation and a second antibody preparation, conjugated to HRP, were obtained for the assay. The enzyme substrate was OPD, supplied as 2mg tablets. RBP standard was supplied as concentrate of 0.5mg/ml. Antibodies, enzyme substrate and RBP standard were purchased from DakoCytomation Ltd. (Ely, Cambridgeshire, UK). The RBP standard was calibrated against a second standard (concentration 11.36mg/ml) supplied by Dr. M. Topping (Occupational Medicine and Hygiene Laboratory, Health and Safety Executive, London, UK).

Analytical reagents used in the preparation of buffers and for other chemical reactions were purchased from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK). All solutions were made using deionised water. Standard laboratory equipment, including single and multiple channel pipettes, centrifuges, mixing equipment and glassware was available at the Wellchild Laboratory, Guy's Hospital, London.

2.2.1.3.3 Experimental protocol

The first antibody solution was prepared by making a 1:1,000 dilution of unconjugated antibody in *coating buffer* [composition: Na_2CO_3 (anhydrous) 25mM; $NaHCO_3$ 25mM; pH 9.6]. All except the top two wells of the NUNC plate were coated with 100µl aliquots of the first antibody solution using an eight-channel pipette. The plate was then covered and incubated overnight at 4°C. On completion of the overnight incubation the antibody solution was discarded and the plate washed using *wash buffer* [composition: NaH_2PO_4 2.5mM; Na_2HPO_4 7.5mM; NaCl 145mM; Tween (polyoxyethylene sorbitan monolaurate) 0.1%; pH 7.2]. The washing step consisted of applying 150µl wash buffer to all wells and then discarding the wash solution using an automatic plate washing device. This process was repeated 3 times.

The RBP standards were freshly prepared from the 0.5mg/ml concentrate, stored at -70°C and thawed for use on the day of the assay. The RBP standard concentrate was diluted in *assay buffer* [composition: NaH_2PO_4 2.0mM; Na_2HPO_4 8.0mM; NaCl 140mM; Tween 0.5%; pH 7.2] to produce a solution of 50µg/l. Further doubling dilutions produced a total of eight RBP standards, with a working range from 0.4 to 50µg/l.

Urine samples were diluted 1:20, 1:200 and 1:2,000 in assay buffer in order to ensure that the estimated RBP concentrations were within the working range of the assay. Three quality controls (QC), one of which was plain assay buffer, were included for every RBP assay. Each of the prepared standards, QC's and samples were added to the antibody-coated plate in 100µl aliquots in duplicate. The top two wells of the plate were left blank. The plate was then incubated at room temperature for two hours after which the contents were discarded and the plate washed as described above.

A solution of second antibody was prepared by mixing the HRP-conjugated antibody with the RBP assay buffer in 1:1,000 dilutions. This solution was applied to the washed plate in aliquots of 100µl per well using an eight channel pipette, except for the top two wells. The plate was further incubated for one hour at room temperature, following which the contents were discarded and the plate washed as before.

A solution of enzyme substrate was prepared twenty minutes before use by adding 10mg of OPD in 15ml *substrate buffer* [composition: Na_2HPO_4 79mM, Citric acid 27mM; Tween 0.1%; pH 6.0], mixing until dissolved and then adding 6.25µl of 30% hydrogen peroxide (H_2O_2) five minutes before use. The substrate solution was added to each well in 100µl aliquots, this time including the top two wells. The plate was then incubated in darkness for fifteen minutes. The colour reaction was stopped by the addition of 150µl 1M H_2SO_4 to each well in the same order as the substrate, ensuring as far as possible the same reaction time in each well. A measurement of the change in absorbance at 490nm in each well was made with an automated ELISA plate reader (Dynatech MR600), and a printout was obtained. A reference wavelength of 630nm

was used to reduce interference from the plate itself. The plate was blanked to zero on the top two wells.

2.2.1.3.4 Data analysis and presentation of urinary RBP concentration

Absorbances for each RBP standard were used to define a sigmoid-shaped standard curve. A composite standard curve for the RBP assay is shown in *figure 2.15*. Sample RBP concentrations were determined from this by the method of linear interpolation using a custom-made spreadsheet. Samples whose mean absorbances fell outside the limits of the standard curve were repeated in a subsequent assay at different concentrations. In order to minimise the effect of variation in free water excretion by the kidney, RBP concentrations were expressed as a ratio to urine creatinine concentration (μg of RBP per mmol of creatinine).

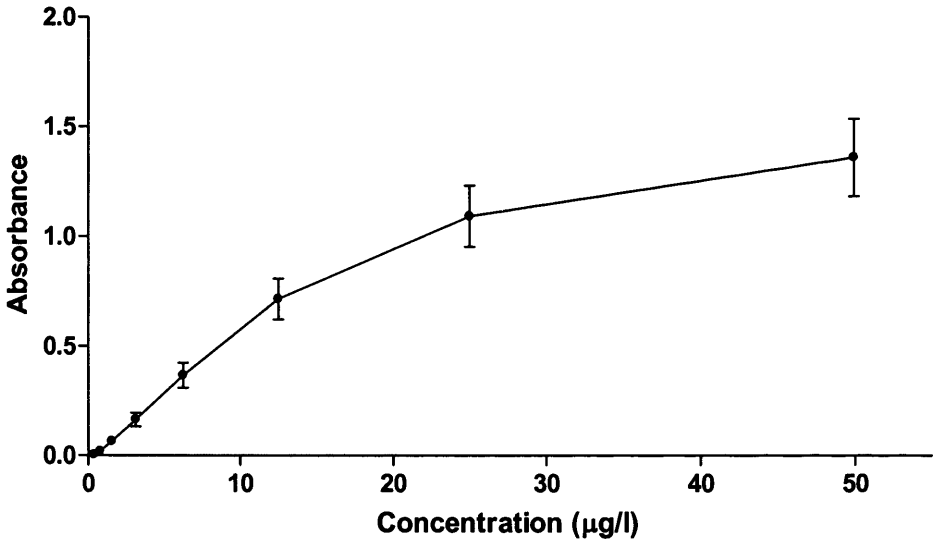


Figure 2.15 Composite standard curve for RBP ELISA displaying mean (standard deviation) absorbance for 10 consecutive assays.

2.2.1.4 Accuracy and reproducibility of the technique

The *detection limit* for the "double-sandwich" RBP ELISA described above was determined by Tomlinson *et al* (482), and was reported to be 0.25µg/l. This is the smallest RBP concentration in a urine sample that can be distinguished from a suitable blank with reasonable probability and is a measure of the sensitivity of the assay (386). The above value is similar to the detection limits reported for other RBP ELISA assays (0.2-5µg/l) (228;316;483).

Recovery is a measure of accuracy of an analytical method and is defined as the ability of an assay to correctly measure pure analyte when added to samples routinely analysed (386). Recovery for the RBP ELISA was determined by Tomlinson *et al* (482) by adding known quantities of RBP protein standard to samples of urine, adding the same volume of assay buffer to other aliquots of the same samples and measuring the test and baseline concentrations. The ratio of the amount recovered to the original quantity added was expressed as a percentage (the ideal being 100%) and for the RBP ELISA was found to range between 103% and 112%.

The intra- and inter-assay coefficients of variation for the RBP ELISA were determined to be 4.2% and 11.9% respectively (concentration range 0.5 to 50µg/l) (482).

2.2.2 Measurement of plasma and urine creatinine by isotope dilution mass spectrometry (ID-MS)

In the clinical study presented in *chapter 8* in this thesis, creatinine concentration was measured in plasma and urine from paediatric renal transplant patients. Plasma concentration of creatinine was used to assess renal function following transplantation. Urinary creatinine values were used for the expression of urine RBP concentration, as described in *section 2.2.1.3.4.* Creatinine concentration was measured by stable isotope dilution mass spectrometry (ID-MS), a modified chromatographic isotope dilution electrospray tandem mass spectrometry method, previously described by Lamb *et al* (274). ID-MS has been proposed as the reference method for creatinine concentration measurement (283;515).

2.2.2.1 Rationale of ID-MS

Isotope dilution mass spectrometry (ID-MS) is the technique of choice for most common clinical analytes in biological samples, providing high analyte specificity and sensitivity with accurate and precise quantitation (134). The use of ID-MS for the determination of the concentration of organic analytes is based on adding a known amount of stable isotope labelled version of the analyte ("spike" solution) as an internal standard, equilibrating the labelled analyte with the endogenous analyte of interest in the sample, and then measuring the ratio of unlabelled to labelled analyte using mass spectrometry. A standard curve of analyte concentration against stable isotope ratio is prepared using standard material allowing analyte concentrations in samples to be accurately determined. A chromatography (liquid or gas) step is usually

included prior to mass spectrometry in order to separate labelled and unlabelled analyte from the sample matrix (140;241).

Mass spectrometry (MS) has evolved to become an irreplaceable technique in the analysis of biological compounds (164). It is a chemical analysis technique that is used to measure the mass of unknown molecules by *ionising*, *separating* and *detecting* ions (*figure 2.16)* according to their mass to charge ratios *(m/z)* (106). This principle can be utilized to determine the identity and amount of analytes in an unknown sample from the type and abundance of ions produced.

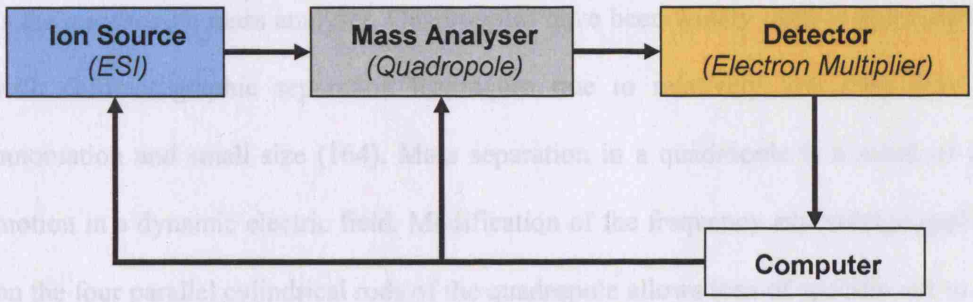


Figure 2.16 Basic components *(examples)* of a typical mass spectrometer. *ESI: electrospray ionisation.*

Given the dependence of MS on the production of ions, the most important reaction in the process is the one that converts analytes of interest into gas-phase ions. Although limitations in initial ionisation approaches (e.g. electron ionisation) restricted the applicability of mass spectrometry in the analysis of biological specimens (164), the introduction of *electrospray ionisation (ESI)* (532) now allows simple conversion of most organic compounds into gas-phase ions. Ions in ESI are generated by passing a solution-based sample though a small capillary, in the presence of an electric field

(164). Electrostatic spraying initially generates an aerosol of charged droplets, which subsequently explode, due to mutual repulsion of charges, leading to the formation of ions. One of the most important limitations of ESI is its susceptibility to *ion suppression effects*. Biological samples are complex mixtures of compounds, and ion formation by the analyte of interest may be suppressed by ionisation of higher concentration analytes. This can be avoided by chromatographically separating analytes of interest from the sample matrix prior to ionisation (140;164).

Following ESI, ions enter the mass analyser, which separates ions according to their *m/z*. Several types of mass analyser have been developed, the most popular of which is the *quadropole* mass analyser. Quadropoles have been widely used in combination with chromatographic separation techniques due to relatively low cost, ease of automation and small size (164). Mass separation in a quadropole is a result of ion motion in a dynamic electric field. Modification of the frequency and voltage applied on the four parallel cylindrical rods of the quadropole allows ions of specific *m/z* to go through the analyser, whereas other ions are retained (164). Ions of interest are then detected by an *electron multiplier* detector that converts the kinetic energy of ions to a current, which is then amplified and recorded.

In ID-MS assays in this thesis, spectrometry was performed using the technique of *tandem* MS (MS/MS), due to its rapid analysis potential and increased sensitivity (73;85;504). MS/MS involves two stages of MS *(figure 2.18)*. The first stage (MS_1) acts as a chemical filter and results in the isolation of ions of a desired *m/z* from the rest of the ions emanating from the ion source. These isolated ions *(parent ions)* are then induced to undergo a reaction that changes either their mass *(m)* or charge *(z)*.

Typically the reactions involve some type of process to increase the internal energy of ions, leading to dissociation (164). The most widely used dissociation method is *collision-induced dissociation (CID)*, in which the parent ion collides with a neutral target (collision) gas (e.g. high purity N_2 gas) and some of the kinetic energy of the parent ion is converted to internal energy (330). The ions resulting from CID are termed *product* ions and these are analysed in the second stage of MS (MS_2) *(figure 2.17)*. MS_2 results in the isolation of a product ion of desired *m/z*, which is subsequently detected and quantified. The MS/MS instrument used in this thesis is a triple quadropole (QqQ) mass spectrometer (542). In the QqQ instrument the first and third quadropoles are operated as mass analysers for MS_1 and MS_2 respectively, whereas the second (middle) quadropole acts as the collision region for the generation of product ions by CID.

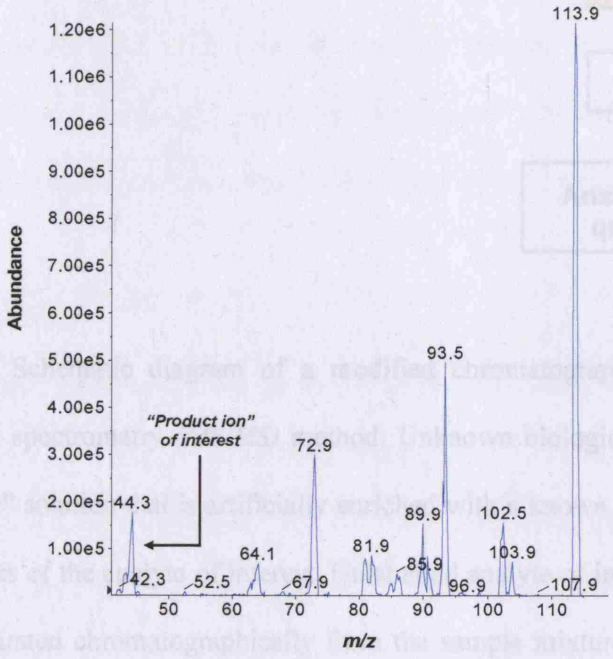


Figure 2.17 Mass spectrum of "product ions" formed by CID of a selected creatinine "parent ion" (*m/z* 114.3). In creatinine assays in this thesis, product ions with *m/z* 44.3 were selected by MS_2 and subsequently detected and quantified.

Figure 2.18 summarises the basic principles of the modified ID-MS assay used in this thesis.

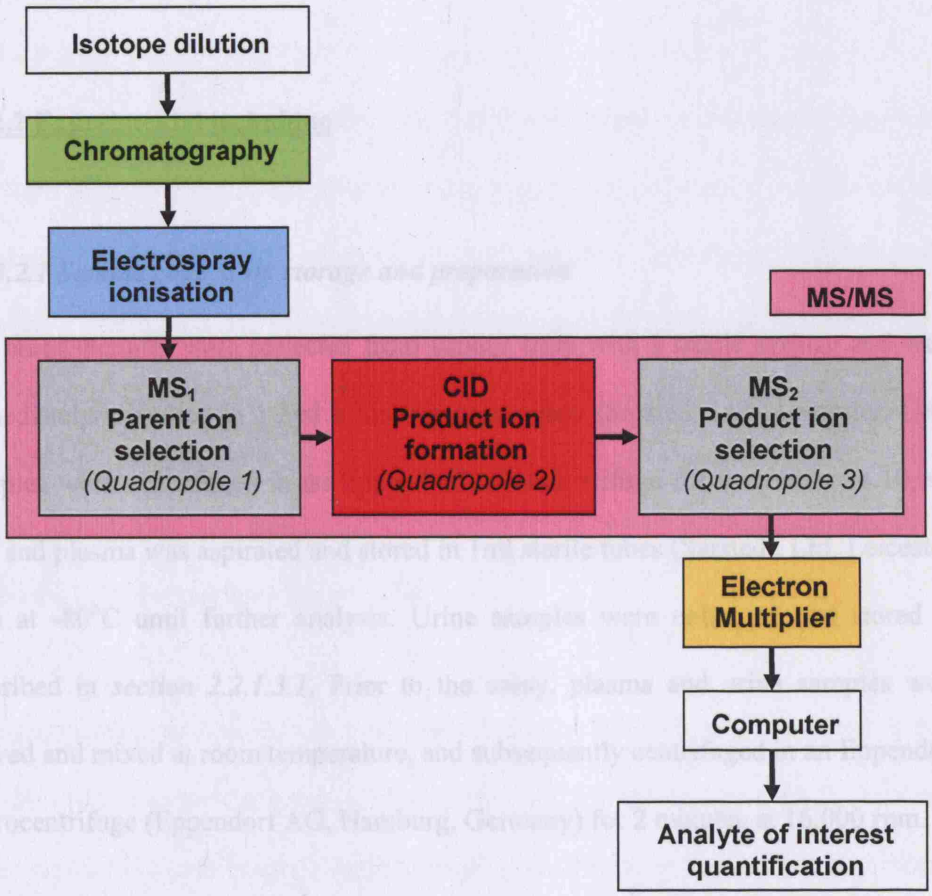


Figure 2.18 Schematic diagram of a modified chromatographic isotope dilution tandem mass spectrometry *(MS/MS)* method. Unknown biological sample is diluted with a "spike" solution that is artificially enriched with a known concentration of one of the isotopes of the analyte of interest. Unlabelled analyte of interest and its isotope are then separated chromatographically from the sample mixture and are ionised by electrospray ionisation *(ESI)*. Parent ions for the analyte and its isotope are selected in the first MS stage *(MS_1)* of MS/MS and are subsequently induced to dissociate into product ions by collision-induced dissociation *(CID)*. Specific product ions for

analyte and isotope are finally selected and their abundance is recorded. The concentration of the analyte of interest is calculated by the ratio of abundance of the analyte/isotope product ions.

2.2.2.2 Experimental technique

2.2.2.2.1 Sample collection, storage and preparation

4ml blood samples were collected from venous lines with a sterile syringe and were immediately aliquoted in 1.2ml lithium-heparin tubes (Sarstedt Ltd., Leicester, UK). Samples were centrifuged in an Eppendorf microcentrifuge for 6 minutes at 10,000 rpm and plasma was aspirated and stored in 1ml sterile tubes (Sarstedt, Ltd, Leicester, UK) at -80°C until further analysis. Urine samples were collected and stored as described in *section 2.2.1.3.1.* Prior to the assay, plasma and urine samples were thawed and mixed at room temperature, and subsequently centrifuged in an Eppendorf microcentrifuge (Eppendorf AG, Hamburg, Germany) for 2 minutes at 16,000 rpm.

2.2.2.2.2 Materials

The assays were performed using 96 deep well polypropylene plates, purchased from Semat Technical Ltd. (St, Albans, Hertfordshire, UK). Creatinine for the preparation of standard solutions (calibrators) was obtained from BDH Chemicals Ltd. (Poole, Dorset, UK). D_3-creatinine (N-methyl-d_3, >98% atom D) was used as internal standard and was purchased from Cambridge Isotopes (Cambridge, Massachusetts, USA). All other analytical reagents were purchased from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK) and all solutions were prepared using deionised water. All

standard laboratory equipment was available at the WellChild Laboratory, Guy's Hospital, London.

2.2.2.2.3 Experimental protocol

Plasma samples (50µl) were pipetted into 1.8ml polypropylene microcentrifuge tubes and were diluted with 50µl deionised water containing 12.5nmol d_3-creatinine, and 200µl acetonitrile, using an Eppendorf Multipipette Plus (Eppendorf AG, Hamburg, Germany). The tubes were capped, vortex mixed, and centrifuged at 16,000rpm for 2 minutes, in an Eppendorf microcentrifuge (Eppendorf AG, Hamburg, Germany). The supernatants (250µl) were transferred into polypropylene deep-well plates, covered with a sealing mat, and transferred to a refrigerated HTS PAL auto-sampler (CTC Analytics, AG, Switzerland) for analysis.

Prepared plasma samples (1µl) were automatically injected into a solvent stream [composition: acetonitrile:water, 1:1 (v/v) with 0.025% (v/v) formic acid], delivered by an Agilent 1100 series quaternary pump (Agilent Technologies Ltd., West Lothian, UK). The pump was programmed to give a flow rate of 250µl/min. Separation of creatinine and d_3-creatinine from the sample matrix was achieved by use of a teicoplanin guard column (ASTEC Ltd., Congleton, Cheshire, UK). Creatinine and d_3-creatinine co-eluted with a retention time of 1.5 minutes.

Urine samples (10µl) were automatically diluted with 990µl deionised water into polypropylene deep-well plates using a Hamilton Microlab Duo system. The diluted samples (5µl) were further diluted into polypropylene deep-well plates with 495µl of deionised water containing 500pmol of d_3-creatinine using the Hamilton Microlab

Duo system. The plates were covered with a sealing mat and transferred to a refrigerated HTS PAL auto-sampler (CTC Analytics AG, Switzerland) for analysis.

Prepared urine samples (2µl) were automatically injected into a solvent stream [composition: acetonitrile:water, 1:1 (v/v) with 0.025% (v/v) formic acid], delivered by an Agilent 1100 series quaternary pump (Agilent Technologies Ltd., West Lothian, UK). The pump was programmed to give a flow rate of 200µl/min. Separation of creatinine and d_3-creatinine from the sample matrix was achieved by use of a Symmetry C8 liquid chromatography column (3.5µm bead size, 2,1x50mm; Waters Corporation, UK). Creatinine and d_3-creatinine co-eluted with a retention time of 0.6 minutes.

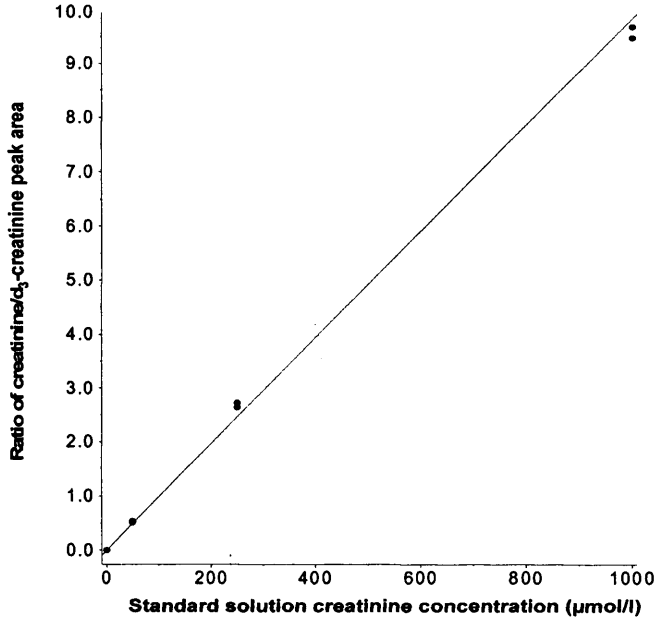
MS/MS was performed using a SCIEX API4000 triple quadropole mass spectrometer, with an ESI source (Applied Biosystems, Warrington, UK). The ESI source was operated in a positive ion mode with a capillary voltage of 5.25kV. Other MS/MS parameters were tuned to maximise the *m/z* transition from 114.3 (parent ion) to 44.3 (product ion) and from 117.3 to 47.3 for creatinine and d_3-creatinine respectively.

Calibration was performed by using standard creatinine solutions. A stock solution was prepared by dissolving creatinine in 0.1mmol/l hydrochloric acid. Working standards for the plasma assay, 50, 250, and 1000µmol/l *(figure 2.19a)*, and urine assay, 1 and 10mmol/l *(figure 2.19b)*, were prepared from the stock standard using deionised water. Creatinine standards were analysed with each assay as described above for plasma and urine samples. Appropriate plasma and urine quality controls were also included with each assay.

2.2.2.2.4 Data analysis and presentation of plasma and urine creatinine concentration

Creatinine calibration plots were generated by plotting the ratio of the MS/MS peak area for creatinine and d_3-creatinine against standard solution creatinine concentration. Composite calibration plots for plasma and urine creatinine assays are shown in *figure 2.19*. The ratio of MS/MS peak area for creatinine and d_3-creatinine was calculated from chromatograms obtained for plasma *(figure 2.20)* and urine samples *(figure 2.21)*, and was then used to determine actual creatinine concentrations from calibration plots. All results were calculated using Analyst, version 1.3.1 (Applied Biosystems, Warrington, UK). Plasma creatinine concentrations were expressed in μmol/l and urine creatinine concentrations were expressed in mmol/l.

a.



b.

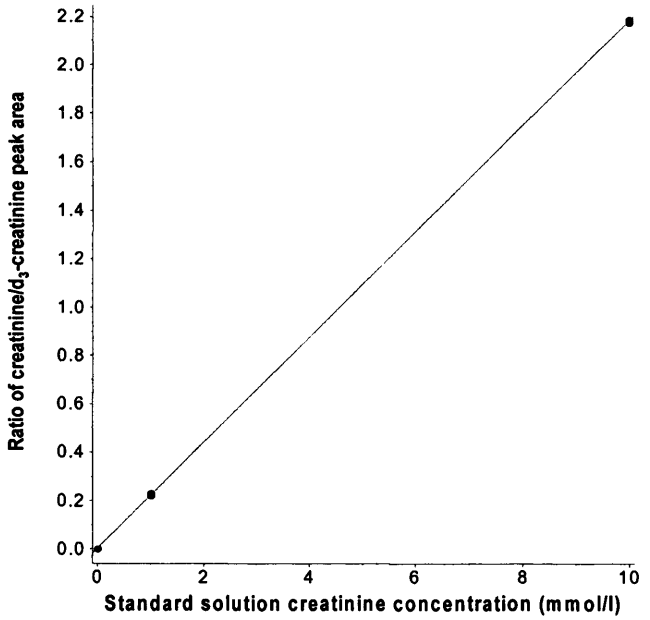


Figure 2.19 Representative calibration plots for plasma (a) and urine (b) creatinine assays.

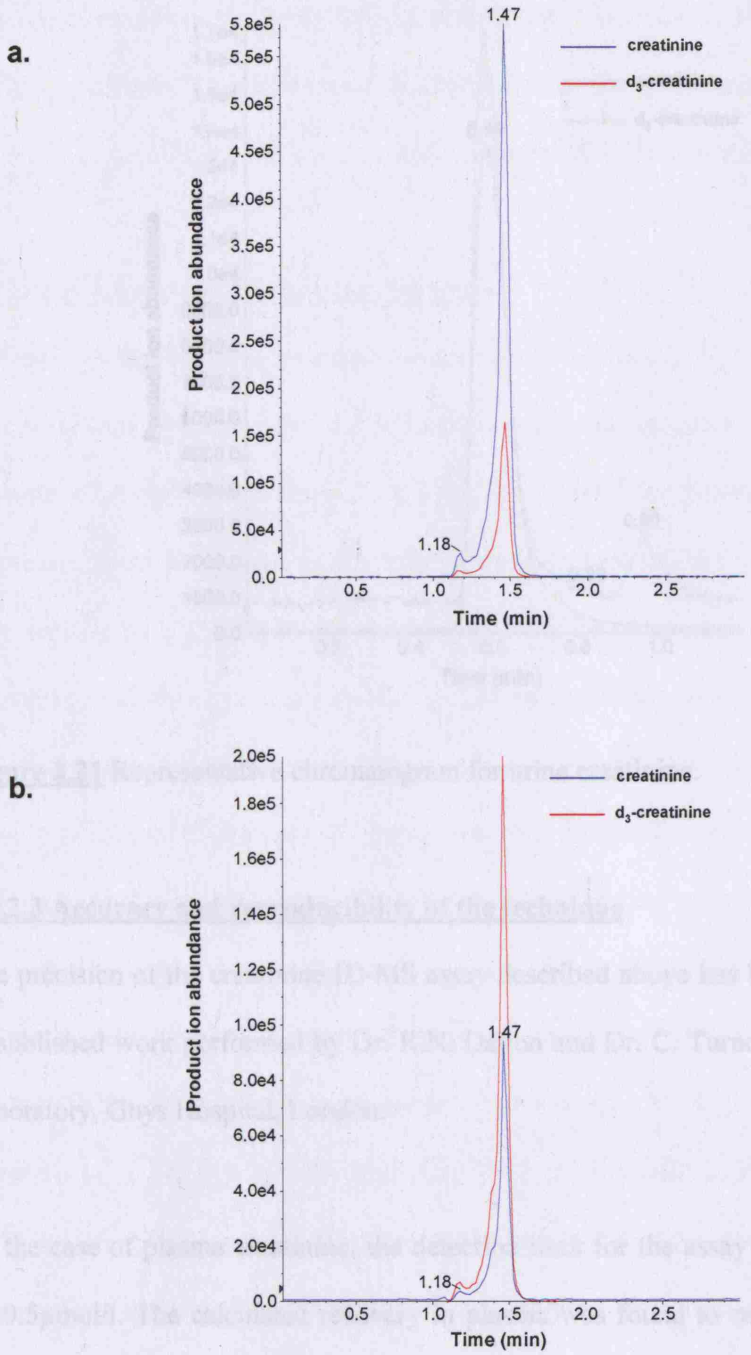


Figure 2.20 Representative creatinine chromatograms from plasma samples with high (a) and low (b) creatinine concentration. The ratio of creatinine (blue) to stable isotope (d_3-creatinine; red) peak area was used to calculate sample creatinine concentration.

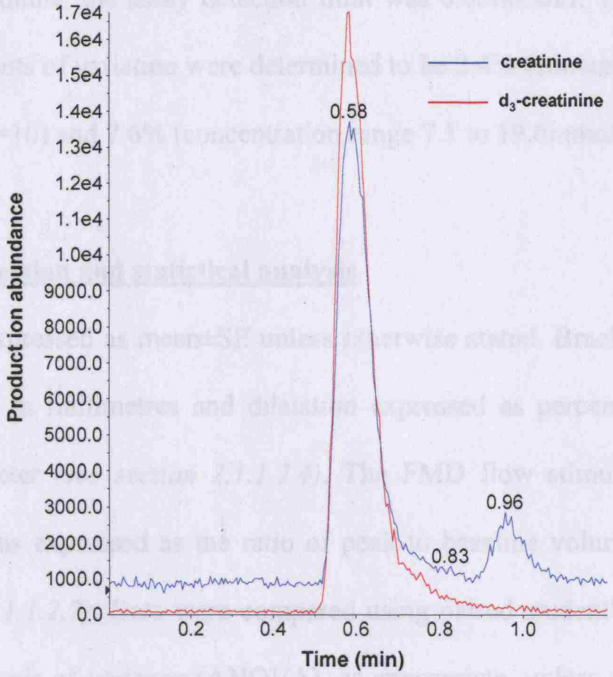


Figure 2.21 Representative chromatogram for urine creatinine.

2.2.2.3 Accuracy and reproducibility of the technique

The precision of the creatinine ID-MS assay described above has been determined in unpublished work performed by Dr. R.N. Dalton and Dr. C. Turner, at the Wellchild Laboratory, Guys Hospital, London.

In the case of plasma creatinine, the detection limit for the assay was determined to be 0.5µmol/l. The calculated recovery in plasma was found to range from 102% to 110%. The intra- and inter-assay coefficients of variation were 3.3% (concentration range 27.4 to 462.9µmol/l; n=10) and 5.6% (concentration range 91 to 508µmol/l; n=52) respectively.

For urine creatinine, the assay detection limit was 0.05mmol/l. The intra- and inter-assay coefficients of variation were determined to be 2.4% (concentration range 1.1 to 10.4mmol/l; n=10) and 7.6% (concentration range 7.1 to 19.6mmol/l; n=378).

2.3 Data expression and statistical analysis

All data are expressed as mean±SE unless otherwise stated. Brachial artery diameter was measured in millimetres and dilatation expressed as percentage increase from baseline diameter *(see section 2.1.1.2.4)*. The FMD flow stimulus during reactive hyperaemia was expressed as the ratio of peak to baseline volume-flow per minute *(see section 2.1.1.2.7)*. Data were compared using paired student's *t*-test or repeated measures analysis of variance (ANOVA), as appropriate, unless otherwise stated. In all studies significance was defined as α-value of less than 5%, indicating that the chance of the null hypothesis still being true even though the difference is greater than the critical value was less than 5%. In the case of studies where the means of more than two variables were compared by t-tests a Bonferroni correction was applied. This states that when 3 or more variables are considered the resultant "p value" was multiplied by all possible comparisons. For example, if 3 groups were used the outcome of A vs. B would be multiplied by 3 (as it would be possible to compare A:B, A:C and B:C) and only if the result was still less than 5% it would be considered statistically significant. All statistical tests were performed with commercially available software – GraphPad Prism (San Diego, California).

2.4 Ethics

Al studies presented in this thesis were approved by the Institute of Child Health and Great Ormond Street Hospital local research ethics committee *(see Appendix C)*.

Chapter 3:

Characterisation of the Remote Ischaemic Preconditioning Stimulus

3.1 Introduction

RIPC of the limb has been shown to be protective against endothelial IR injury of human resistance vessels *(see section 1.5)* (243). In these studies intermittent ischaemia of the contra-lateral arm (three 5 minute periods of ischaemia each followed by 5 minutes of reperfusion) was used as the preconditioning stimulus. As discussed in *section 1.4.1*, a variety of RIPC stimuli have been used in animal studies to induce protection against IR injury to the myocardium and other tissues. In the case of local ("classic") IPC, the degree of protection afforded depends on the "strength" of the preconditioning stimulus, with evidence of a preconditioning "threshold" that needs to be reached for the protective effects to be manifested (298;341;538). Whether the same is true for RIPC is not clear either in animals or humans. The aim of this chapter was to investigate the characteristics of the RIPC stimulus in healthy volunteers, and establish whether varying the duration, site of application of the stimulus or volume of tissue made ischaemic has an effect on the degree of protection against endothelial IR injury.

Because of the invasive nature of the approach necessary to investigate the forearm resistance vascular bed (the brachial artery needs to be cannulated to infuse endothelial stimulants) I chose to use a non-invasive approach to assess flow-mediated dilatation of the brachial artery. Amongst the advantages of this approach is the acceptability of this procedure to volunteers, so that studies could be repeated within the same cohort of individuals, limiting variability of results. However, whether RIPC protects conduit vessels has never been determined so a secondary aim of this chapter was to determine the potential protective effects of RIPC against brachial artery endothelial IR injury in humans, *in vivo*.

3.2 Methods

3.2.1 Subjects

101 studies were performed on 24 healthy volunteers (14 men, 10 women; mean age±SD 27.1 ± 7.3 years; range, 18 to 48) who gave informed consent. Volunteer exclusion criteria are described in *section 2.1.1.2.1.* Studies were performed in a temperature-controlled laboratory (24° to 26°C) and all studies repeated in the same volunteers were at least 7 days apart.

3.2.2 Induction of Ischaemia-Reperfusion (IR)

The non-dominant forearm was made ischaemic by inflating a 9-cm-wide blood pressure cuff placed around the upper arm. The cuff was inflated to a pressure of 200 mm Hg for 20 minutes (index ischaemia), after which it was deflated allowing the arm to reperfuse.

3.2.3 Induction of Remote Ischaemic Preconditioning (RIPC)

Remote ischaemic preconditioning (RIPC) was induced by inflating a 9-cm-wide blood pressure cuff placed around the upper part of: *(i)* the *contralateral* arm, or *(ii)* the *contralateral* forearm (immediately below the antecubital fossa), or *(iii)* the *contralateral* leg (immediately below the femoral region). The cuff was inflated to 200 mm Hg for 5 minutes (ischaemia of the arm, forearm, or leg respectively), followed by a 5-minute deflation. The inflation/deflation cycle constituted a single RIPC cycle.

3.2.4 Assessment of Conduit Vessel Function

Endothelial function of the brachial artery was assessed by flow-mediated dilation (FMD) of the brachial artery in the non-dominant arm, as described in *section 2.1.1.2.* The dilator response of the brachial artery to glyceryl trinitrate (GTN; 25μg sublingually) was used to assess endothelium-independent dilation *(section 2.1.1.3).*

3.2.5 Experimental protocols

3.2.5.1 Effect of IR on conduit vessel vascular dilator function

In order to determine the effect of IR on endothelial function, FMD was assessed before ischaemia and at 20 minutes after reperfusion (n=11; *figure 3.1, protocol a*). Similarly, the effect of IR on smooth muscle function was determined in separate studies, by assessing the dilation in response to sublingual GTN (25μg) before and after IR. NO donors have been shown to offer preconditioning-like protection against IR injury (202;289), so I performed pilot experiments to assess whether GTN had direct effects to reduce IR injury to the brachial artery *(see section 3.2.5.4).* These studies indicated that GTN protected against IR-induced endothelial dysfunction when administered immediately before, but not 24 hours before IR *(section 3.3.1).* Therefore, to assess whether IR altered the dilator response to GTN, the control dilatation to GTN was measured 24 hours before IR and compared with the dilation to GTN immediately after IR (n=7; *Figure 3.1, protocol b*).

3.2.5.2 Characterisation of the stimulus required for induction of protection by RIPC against endothelial IR injury

FMD was assessed before and after IR immediately preceded by RIPC. To test the effect of varying the number of RIPC cycles and the volume of tissue preconditioned

on the induction of preconditioning, the following RIPC stimuli were used: *(i)* 3 RIPC cycles applied on the arm (RIPC*Arm3C*; n=9; *figure 3.1, protocol c*); *(ii)* 2 RIPC cycles applied on the arm (RIPC*Arm2C*; n=7; *figure 3.1, protocol d*); *(iii)* 3 RIPC cycles applied on the leg (RIPC*Leg3C*; n=9; *figure 3.1, protocol c*); *(iv)* 2 RIPC cycles applied on the leg (RIPC*Leg2C*; n=9; *figure 3.1, protocol d*); 3 RIPC cycles applied on the forearm (RIPC*Forearm3C*; n=7; *figure 3.1, protocol c*); 5 RIPC cycles applied on the forearm (total duration of stimulus 50 minutes) (RIPC*Forearm5C*; n=6; *figure 3.1, protocol e*). In control studies brachial artery FMD was measured before and after RIPC (3 cycles on the contralateral arm) alone followed by 40 minutes of rest (instead of 20 minutes of ischaemia and 20 minutes of reperfusion) to determine whether RIPC had a direct effect on endothelial function (n=7).

3.2.5.3 Exploratory analysis to investigate the "dose-response" characteristics of RIPC

Post-IR FMD values for each of the RIPC protocols *(section 3.2.5.2)* were expressed as a ratio (%) to baseline FMD *("RIPC protection index")*. The RIPC protection index is a measure of the protective potential of RIPC against IR, and its value decreases (from a maximum of 100%) with increasing injury (i.e. decreasing RIPC-induced protection) to the endothelium. In order to establish whether the degree of protection by RIPC depends on the "strength" of the preconditioning stimulus, RIPC protection index values for the various RIPC stimuli were compared to each other and to the post-IR to baseline FMD ratio from control studies (IR alone).

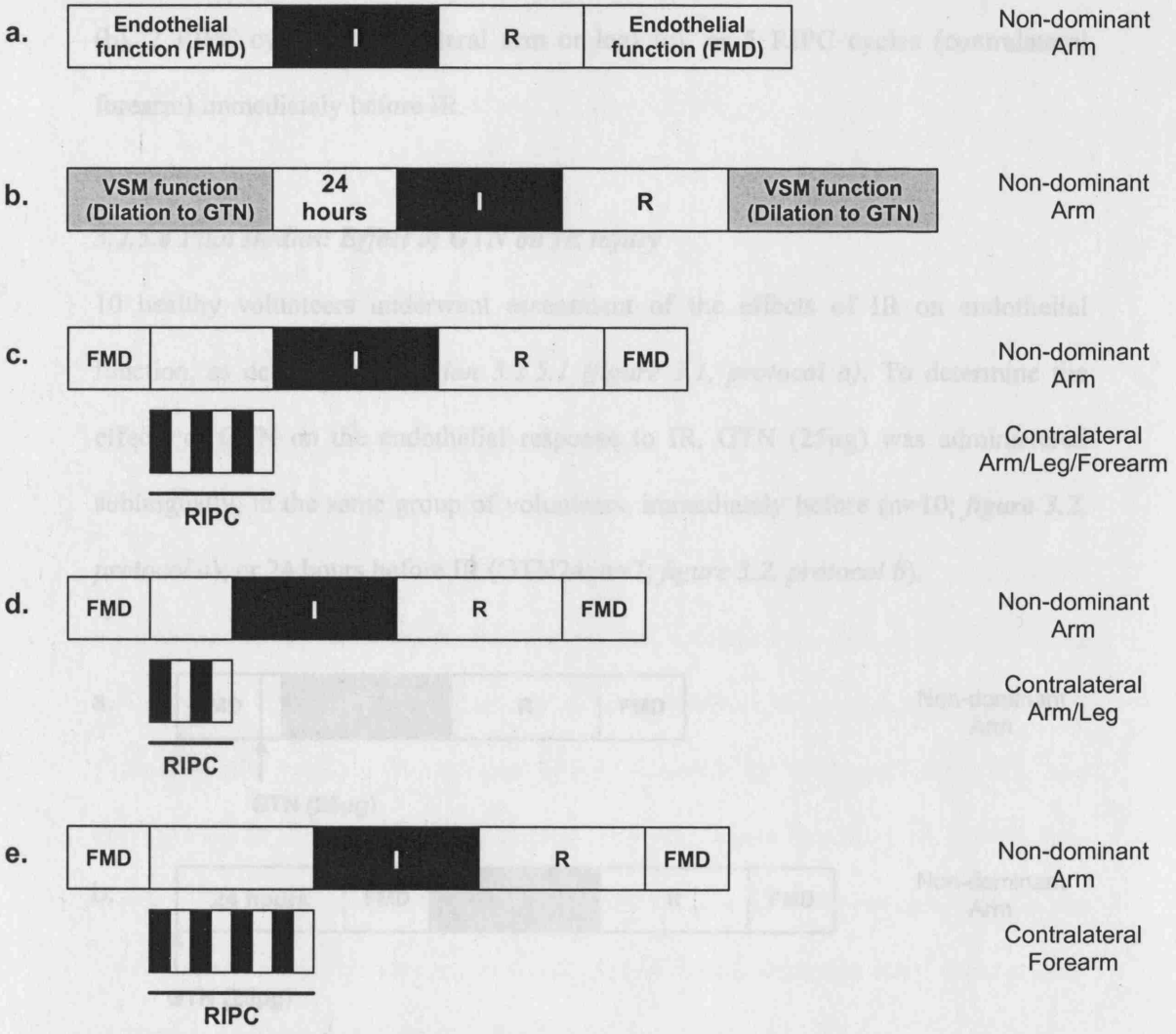


Figure 3.1 Protocol of studies to determine the nature of the stimulus required for protection by RIPC. Brachial artery endothelial function was assessed by flow-mediated dilation (FMD) before 20 minutes of arm ischaemia (I) and at 20 minutes of reperfusion (R) (a). The effect of IR on brachial artery smooth muscle function was determined by measuring dilation in response to sublingual glyceryl trinitrate (GTN; 25μg) administered 24 hours before and immediately after IR (b). The effect of remote ischaemic preconditioning (RIPC) on endothelial ischaemia-reperfusion (IR) injury was determined by applying 3 RIPC cycles (contralateral arm, leg or forearm)

(b), 2 RIPC cycles (contralateral arm or leg) (c), or 5 RIPC cycles (contralateral forearm) immediately before IR.

3.2.5.4 Pilot studies: Effect of GTN on IR injury

10 healthy volunteers underwent assessment of the effects of IR on endothelial function, as described in *section 3.2.5.1 (figure 3.1, protocol a)*. To determine the effects of GTN on the endothelial response to IR, GTN (25μg) was administered sublingually, in the same group of volunteers, immediately before (n=10; *figure 3.2, protocol a*), or 24 hours before IR (GTN24; n=7; *figure 3.2, protocol b*).

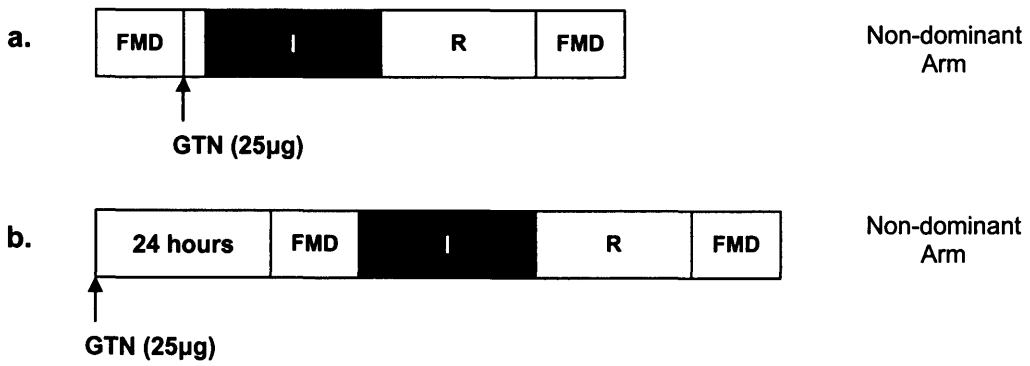


Figure 3.2 Protocol of pilot studies to determine the effect of GTN on endothelial IR injury.

3.2.6 Calculations and Statistics

Data expression and statistical analysis was performed as described in *section 2.3*. For multiple comparisons (3 or 7 groups), p values by ANOVA (repeated measures) were Bonferroni adjusted. In all cases, $p<0.05$ was considered statistically significant. A power calculation was performed in order to determine the appropriate sample size for the studies. I calculated that a sample size of 10 subjects will be needed to show that

RIPC increases FMD following IR injury by 30%, based on a within subject standard deviation of 0.9, an α value of 0.05 and a β level of 0.8.

3.3 Results

All subjects tolerated the procedures without any complications.

3.3.1 Pilot studies: Effect of GTN on IR injury

IR resulted in brachial artery endothelial dysfunction in the non-dominant arm (FMD 8.0±1.3% pre- versus 4.9±1.3% post-IR, $p<0.01$; n=10; *figure 3.3a*), but had no effect on brachial artery endothelial function in the contralateral arm. The IR-induced reduction in FMD was prevented by GTN administered immediately before IR (7.8±1.6% pre- versus 8.0±1.2% post-IR+GTN, p=NS; n=10; *figure 3.2b*). GTN had no effect on IR injury when administered 24 hours before IR (FMD 10.1±1.4% pre- versus 5.3±1.6% post-IR+GTN24, $p<0.001$; n=7; *figure 3.2c*). These results could not be explained by differences in pre- and post-IR blood pressure, heart rate, arterial diameter and FMD flow stimulus *(table 3.1)*.

Based on these observations, the protocol for assessing the effects of IR on vascular smooth muscle function *(figure 3.1, protocol b)* was designed to include baseline measurement of brachial artery endothelium-independent dilation in response to GTN 24 hours prior to induction of IR, in order to avoid direct effects of GTN on IR injury *(see section 3.2.5.1)*.

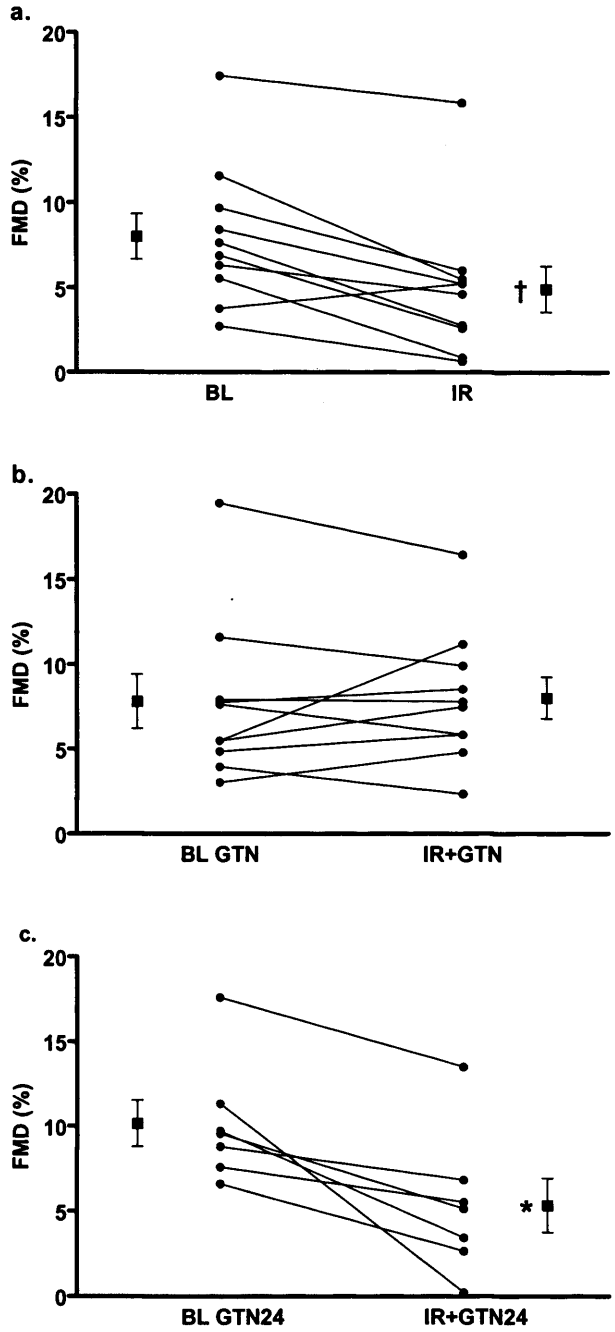


Table 3.3 Results of pilot studies to determine the effect of GTN on IR injury. IR reduced FMD (a; BL 8.0±1.3% vs. IR 4.9±1.3%; † $p<0.01$, ANOVA; n=10). A 25μg-dose of GTN administered immediately before IR prevented IR-induced endothelial dysfunction (b; BL 7.8±1.6% vs. IR+GTN 8.0±1.2%; p=NS, ANOVA; n=10) but had no effect on IR injury when administered 24 hours before IR (c; BL 10.1±1.4% vs. IR+GTN24 5.3±1.6%; * $p<0.001$, ANOVA; n=7).

	IR alone (n=10)		IR+GTN (n=10)		IR+GTN24 (n=7)	
	(A) Pre	(B) Post	(C) Pre	(D) Post	(E) Pre	(F) Post
SBP	114±2	112±2	113±2	114±3	112±2	110±2
DBP	67±2	65±2	66±2	68±2	68±3	68±2
HR	66±5	59±4	61±3	59±3	62±6	59±4
Arterial diameter	4.1±0.3	4.1±0.3	4.1±0.2	4.1±0.1	4.0±0.2	3.9±0.3
Flow stimulus	9.0±2.7	9.1±2.2	8.0±1.4	6.7±1.4	6.1±1.4	8.0±1.3
FMD	**8.0±1.3**	**4.9±1.3 †**	**7.8±1.6**	**8.0±1.2**	**10.1±1.4**	**5.3±1.6 ***

Table 3.1 Summary of pre- and post-IR data from pilot studies to test the effect of GTN on IR injury. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). Flow mediated dilation (FMD) was expressed in peak % dilation from baseline diameter. *† p<0.01 FMD (A) vs. (B) and (B) vs. (D); * p<0.001 FMD (E) vs. (F) (ANOVA).*

3.3.2 Effect of IR on vascular dilator function

IR reduced brachial artery FMD (9.3±1.2% pre- versus 3.3±0.7% post-IR, p<0.001; n=11; *figure 3.4a*) but had no effect on blood pressure, heart rate, brachial artery diameter or FMD stimulus during reactive hyperaemia *(table 3.2a)*. IR had no effect on GTN dilatation (9.3±2.0% pre-IR versus 10.0±2.0%, p=NS; n=7; *figure 3.4b* and *table 3.2b*).

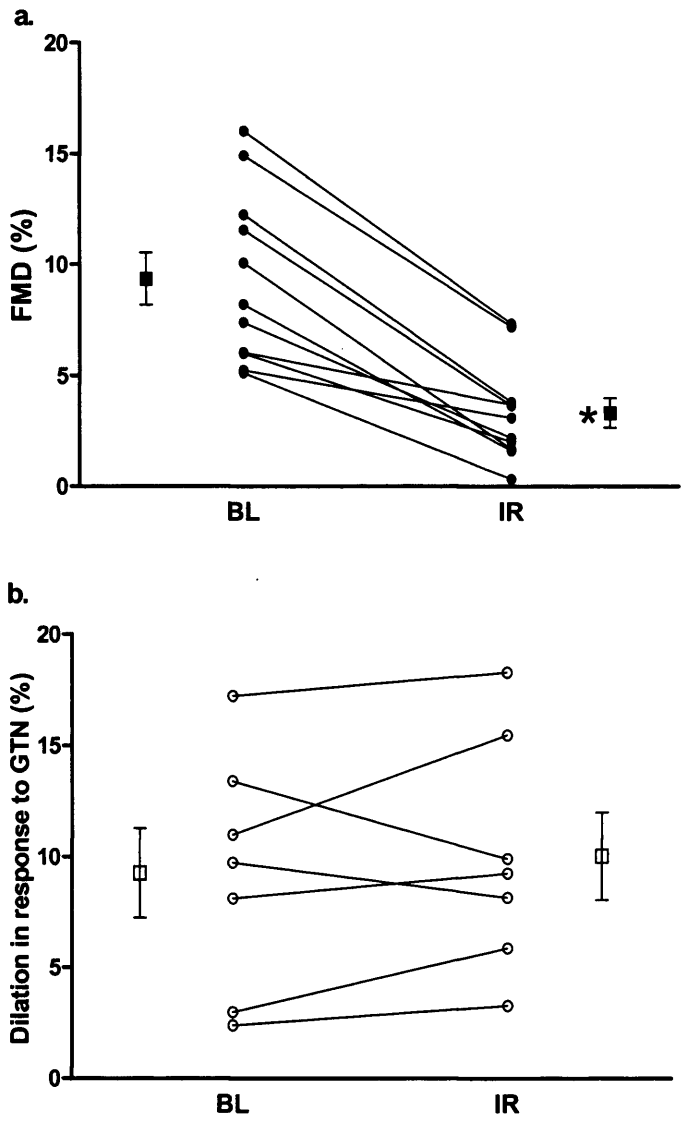


Figure 3.4 Effect of IR on endothelial and smooth muscle function. FMD was 9.3±1.1% at baseline (BL) and was reduced by IR (a; IR 3.3±1.2%; * $p<0.001$ vs. BL, ANOVA; n=11). GTN dilatation was 9.3±2.0% at BL, and was unaffected by IR (b; IR 10.0±2.0%; p=NS, paired *t*-test; n=7).

a.

	IR alone (effect on endothelial function) (n=11)	
	Pre	*Post*
SBP	110±2	110±2
DBP	59±1	60±2
HR	61±2	59±2
Arterial diameter	3.3±0.2	3.3±0.2
Flow stimulus	6.3±1.8	8.0±1.6
FMD	**9.3±1.2**	**3.3±0.7** *

b.

	IR alone (effect on smooth muscle function) (n=7)	
	24 hours pre	*Post*
SBP	110±4	108±4
DBP	64±2	62±2
HR	69±2	69±2
Arterial diameter	3.5±0.2	3.5±0.2
Dilation in response to GTN	**9.3±2.0**	**10.0±2.0**

Table 3.2 Summary of pre- and post-IR data from studies to test the effect of IR on brachial artery endothelial (a) and smooth muscle function (b). SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). Flow mediated dilation (FMD) and the dilator response to GTN were expressed in peak % dilation from baseline diameter. * *$p<0.001$ FMD pre- vs. post-IR (ANOVA).*

3.3.3 Effect of RIPC on the endothelial response to IR

RIPC protocols had no effect on baseline blood pressure, heart rate, brachial artery diameter, and FMD flow stimulus during reactive hyperaemia *(Table 3.3)*

3.3.3.1 RIPC on the arm

3 RIPC cycles applied on the contralateral arm immediately before IR (RIPC*Arm3C*) prevented endothelial dysfunction (FMD 8.6±0.9% pre- versus 7.1±0.9% post-IR+RIPC*Arm3C*, p=NS; n=9; *figure 3.5a*) but had no direct effect on brachial artery FMD (7.6±0.8% pre- versus 7.2±0.9% post-RIPC, p=NS; n=7). In contrast, there was endothelial dysfunction when 2 RIPC cycles were applied on the arm (FMD 8.2±1.2% pre- versus 4.7±1.1% post IR+RIPC*Arm2C*, p<0.01; n=9; *figure 3.5b*).

3.3.3.2 RIPC on the leg

IR-induced endothelial dysfunction was prevented by 3 RIPC cycles (FMD 8.0±1.2% pre- versus 7.6±1.3% post IR+RIPC*Leg3C*, p=NS; n=9; *figure 3.6a*), and 2 RIPC cycles applied on the leg (FMD 8.9±0.9% pre- versus 7.2±0.9% post-IR+RIPC*Leg2C*, p=NS; n=9; *figure 3.6b*).

3.3.3.3 RIPC on the forearm

RIPC applied on the forearm was protective when 5 RIPC cycles were used (FMD 8.7±2.0% pre- versus 7.9±2.1% post-IR+RIPC*Forearm5C*, p=NS; n=7; *figure 3.7b*) but not when 3 cycles were used (FMD 9.7±1.6% pre- versus 5.6±1.3% post-IR, p<0.01; n=6; *figure 3.7a*).

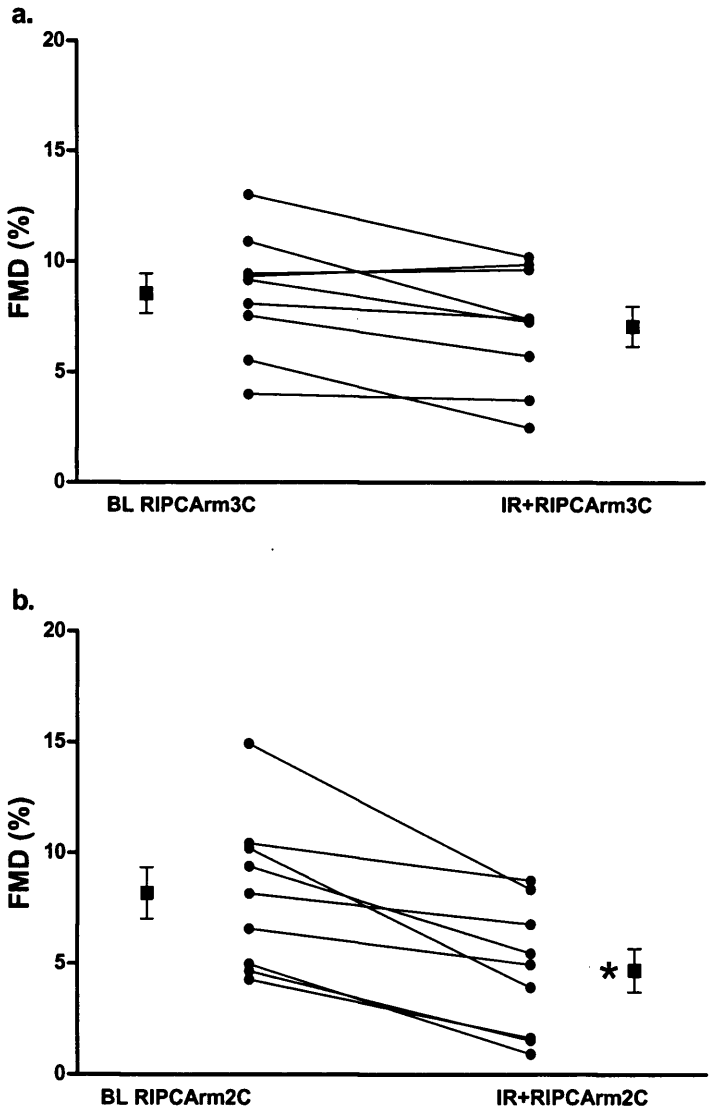


Figure 3.5 Effect of RIPC applied on the arm on endothelial IR injury. 3 RIPC cycles applied on the arm (RIPC*Arm3C*) prevented IR-induced endothelial dysfunction (a; BL 8.6±0.9% vs. IR+RIPC*Arm3C* 7.1±0.9%; p=NS, ANOVA; n=9). Endothelial dysfunction was observed when 2 RIPC cycles were applied on the arm (RIPC*Arm2C*) (b; BL 8.2±1.2% vs. IR+RIPC*Arm2C* 4.7±1.1%; *p<0.001, ANOVA; n=9). *BL: baseline.*

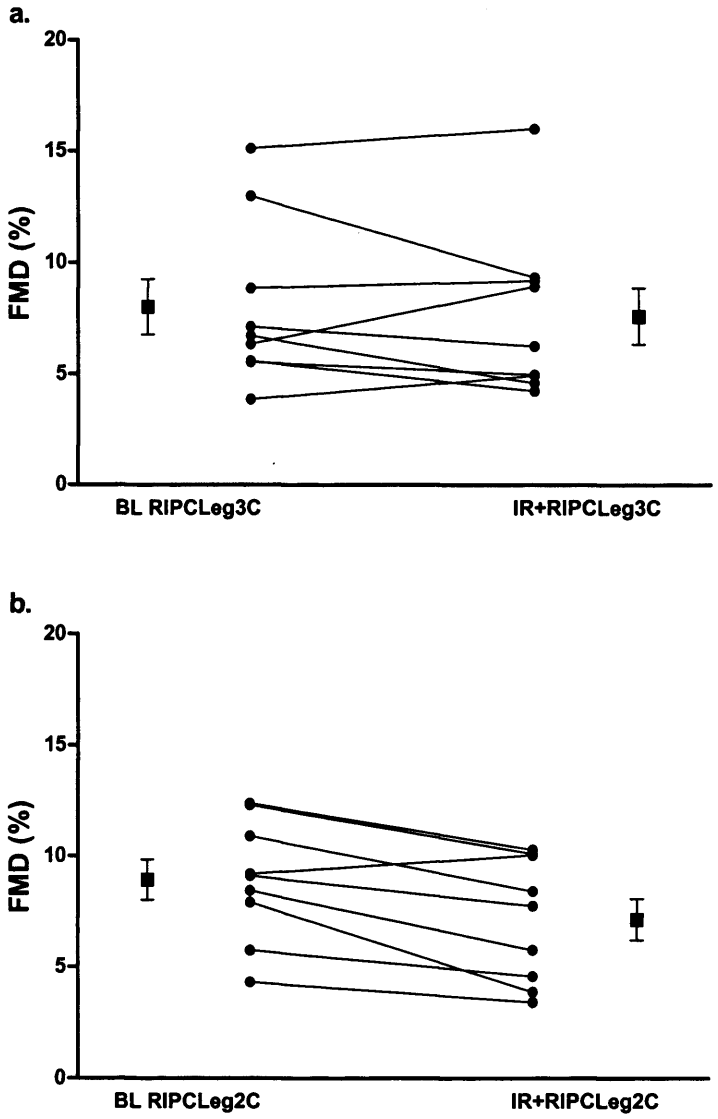


Figure 3.6 Effect of RIPC applied on the leg on endothelial IR injury. A 3-cycle RIPC stimulus applied on the leg (RIPC*Leg3C*) prevented the reduction in FMD due to IR (a; BL 8.0±1.2% vs. IR+RIPC*Leg3C* 7.6±1.3%; p=NS, ANOVA; n=9). In addition, 2 RIPC cycles on the leg were also protective against endothelial IR injury (e; BL 8.9±0.9% vs. IR+RIPC*Leg2C* 7.2±0.9%; p=NS, ANOVA; n=9). *BL: baseline.*

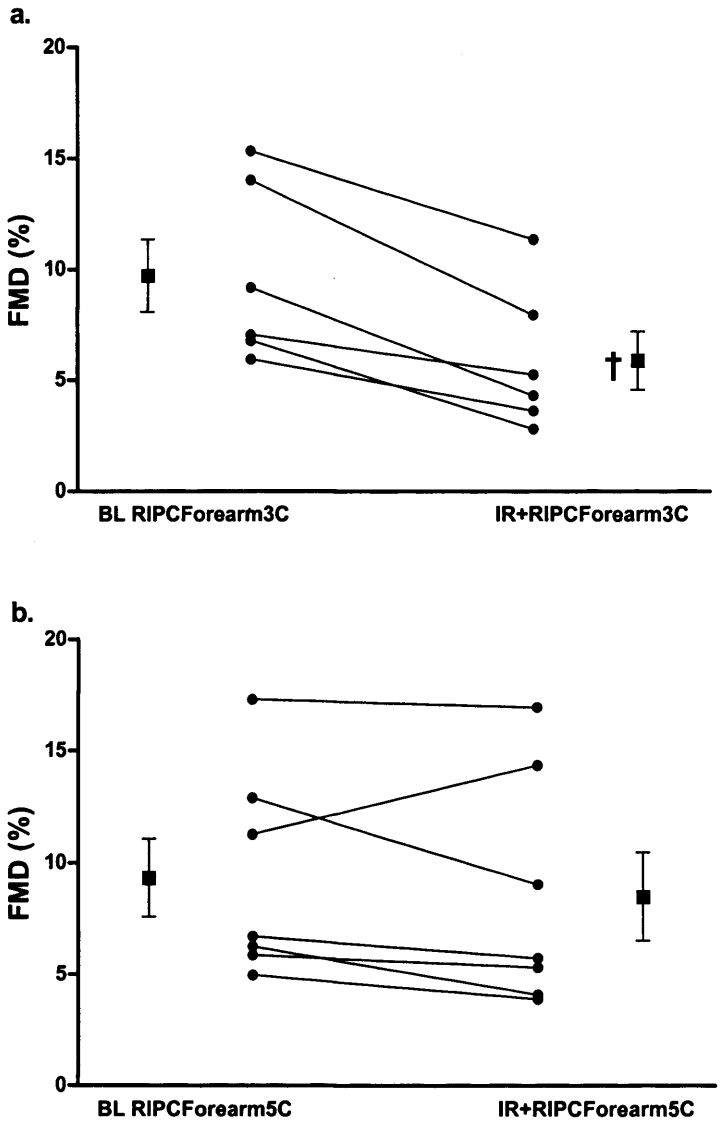


Figure 3.7 Effect of RIPC applied on the forearm on endothelial IR injury. 3 RIPC cycles applied on the forearm did not prevent the IR-induced reduction in FMD (a; BL 9.7±1.6% vs. IR+RIPC*Forearm3C* 5.5±1.3%; † $p<0.01$, ANOVA; n=6). In contrast, when the number of RIPC cycles on the forearm was increased to 5, RIPC resulted in protection against IR (b; BL 8.7±2.0% vs. IR+RIPC*Forearm5C* 7.9±2.1%; p=NS, ANOVA; n=7). *BL: baseline.*

3.3.3.4 "Dose-response" characteristics of RIPC

Figure 3.8 summarises RIPC protection index values for the various RIPC stimuli used in studies in this chapter. IR alone reduced FMD to 34.8±5.2% of its baseline value. RIPC protocols attenuated the post-IR reduction in FMD, with a graded increase in the RIPC protection index with increasing "strength" of the preconditioning protocol. Maximum protection was seen when 3 RIPC cycles were applied on the leg in advance of IR (RIPC protection index 96.8±8.4%, $p<0.001$ versus IR alone).

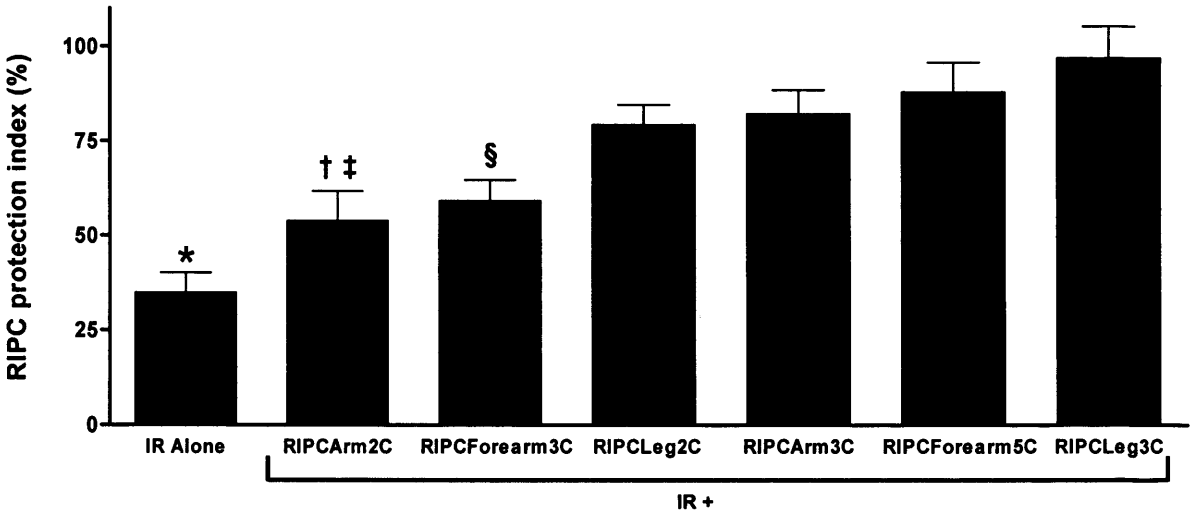


Figure 3.8 "Dose-response" relationship for RIPC. The degree of protection against endothelial IR injury varies with the "strength" of the RIPC stimulus used. *RIPC protection index: ratio (%) of post-IR to baseline FMD; * $p<0.001$ vs. RIPCLeg2C, RIPCArm3C, RIPCForearm5C and RIPCLeg3C; † $p<0.001$ vs. RIPCLeg3C; ‡ $p<0.05$ vs. RIPCArm3C and RIPCForearm5C; § $p<0.05$ vs. RIPCLeg3C.*

	IR+RIPCArm3C (n=9)		IR+RIPCArm2C (n=9)		IR+RIPCLeg3C (n=9)		IR+RIPCLeg2C (n=9)		IR+RIPCForearm3C (n=6)		IR+RIPCForearm5C (n=7)	
	(A) Pre	(B) Post	(C) Pre	(D) Post	(E) Pre	(F) Post	(G) Pre	(H) Post	(I) Pre	(J) Post	(K) Pre	(L) Post
SBP	114±4	115±4	110±2	112±2	112±3	115±4	112±3	110±2	115±4	114±4	114±3	115±3
DBP	60±1	63±3	63±2	63±2	62±2	62±1	60±3	63±1	63±4	62±3	65±2	65±2
HR	62±3	58±3	63±4	57±3	60±3	66±6	65±3	61±3	64±3	60±4	63±4	59±3
Arterial diameter	3.1±0.2	3.3±0.2	3.3±0.2	3.4±0.2	3.4±0.2	3.4±0.2	3.3±0.2	3.3±0.2	3.4±0.3	3.4±0.3	3.4±0.3	3.4±0.3
Flow stimulus	7.5±1.6	7.5±1.7	8.1±1.2	9.5±1.4	11.4±4.0	11.5±3.8	7.7±2.3	9.0±1.9	8.1±2.1	7.4±1.3	6.7±1.6	7.7±2.1
FMD	**8.6±0.9**	**7.1±0.9 ***	**8.2±1.2**	**4.7±1.1 †**	**8.0±1.2**	**7.6±1.3 §**	**8.9±0.9**	**7.2±0.9 †**	**9.7±1.6**	**5.5±1.3 †‡**	**9.3±1.7**	**8.5±2.0 §**

Table 3.3 Summary of pre- and post-IR data from studies designed to determine the ability of various RIPC stimuli to induce protection against IR. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter was measured in millimetres and the FMD flow stimulus during reactive hyperaemia was expressed as the ratio of peak to baseline volume flow per minute (no units). FMD was expressed as peak % dilation from baseline brachial artery diameter. Studies reported in the present table as well as in *table 3.2* were performed sequentially in the same group of volunteers allowing within subject comparisons to be made. ** $p<0.001$ FMD (B) vs. post-IR alone (table 3.2a); † $p<0.01$ FMD (C) vs. (D) and (I) vs. (J); § $p<0.01$ FMD (F) vs. IR alone and (L) vs. IR alone (table 3.2a); ‡ $p<0.05$ FMD (J) vs. IR alone (table 3.2a) (ANOVA).*

3.4 Discussion

This study demonstrates for the first time in humans that RIPC of the limb protects against endothelial IR injury in conduit vessels. Protection by RIPC is determined by the duration of the RIPC stimulus, and the volume of tissue undergoing preconditioning.

Twenty minutes of arm ischaemia followed by reperfusion resulted in endothelial dysfunction in the brachial artery of healthy volunteers, demonstrated by a significant reduction in FMD at 20 minutes of reperfusion that could not be attributed on direct effects of the IR protocol on the FMD flow stimulus during reactive hyperaemia. To establish that the site of IR-induced injury was the vascular endothelium, it was necessary to determine the effects of IR on vascular smooth function, by assessing the dilator response of the brachial artery to the nitric oxide (NO) donor glyceryl-trinitrate (GTN).

However, this posed an obvious problem, because the stimulus used to cause smooth muscle dilatation (NO) might also modulate the response to IR injury. NO has been implicated in the mechanisms of preconditioning *(see sections 1.4.3.1.6 and 1.4.3.3.4)*, with the majority of studies supporting a role for NO as both trigger and mediator of late but not early protection by IPC (54;425;538). Administration of NO donors immediately before or 24 hours before an ischaemic insult resulted in preconditioning-like protection against myocardial IR injury in animals (202;353) and humans (229;289). In addition NO has been demonstrated to have direct effects to reduce IR injury without activating preconditioning mechanisms (52). Therefore, I designed a pilot study to determine whether the dose of GTN used for assessment of

vascular smooth muscle function had any effects on the vascular response to IR. These studies showed that GTN prevents the IR-induced reduction in FMD when administered immediately before but not 24 hours before IR. These observations are consistent with early preconditioning-like protection by exogenous NO, or a direct action to reduce IR injury.

Based on the results of the pilot study, the effects of IR on smooth muscle function were assessed by measuring dilation in response to GTN 24 hours before and immediately after IR, to exclude direct effects of GTN on IR injury. These studies demonstrated that the IR protocol did not affect vascular smooth muscle function, confirming that the observed reduction in FMD was solely due to the effects of IR on the vascular endothelium.

RIPC administered at different sites immediately before arm ischaemia prevented IR-induced endothelial dysfunction. However, some of the stimuli used were not sufficient to achieve protection. Three RIPC cycles on the arm or the leg resulted in a similar degree of protection against IR injury, but a stimulus of reduced duration (two instead of three RIPC cycles) was protective when applied on the leg but not on the arm. These observations suggest that both the duration of the RIPC stimulus, and the volume of tissue undergoing preconditioning have an effect on the strength of the RIPC stimulus. The latter is further supported by the observation that it was necessary to increase the duration of the stimulus (from three to five cycles) in order to achieve protection by RIPC administered on a smaller volume of tissue (forearm). This also explains why the FMD stimulus itself (five minutes of forearm ischaemia) is unlikely

to have a preconditioning effect. None of the RIPC stimuli had direct effects on brachial artery endothelial function.

The present study suggests that RIPC is unlikely to be an "all-or-nothing" phenomenon and appears similar to the graded protective response demonstrated in animal models of RIPC (512). It is possible that there is threshold stimulus below which there is no protective effect, but this remains unproven at present. The various stimuli used in this chapter caused varying degrees of protection, although this effect was too small in some protocols to reach statistical significance. However additional studies would be required to be more certain of this.

3.5 Conclusions

This is the first demonstration of protection by RIPC against endothelial IR injury in human conduit vessels. The present study confirms that RIPC, induced by applying short periods of ischaemia on non-vital tissues results in systemic protection against IR injury, with evidence that development of protection depends on the strength of the preconditioning stimulus. In the next chapter I will present data regarding the time-course of protection from RIPC.

Chapter 4:

Time-course of Protection by

Remote Ischaemic Preconditioning

4.1 Introduction

Animal studies indicate that RIPC offers delayed protection from IR injury that is evident 24 hours following the application of the preconditioning stimulus and lasts for up to 72 (525) to 168 (470) hours *(see section 1.4.2 and table A.9, appendix A).* However, it has not been established whether there are early and late phases of protection (as for IPC) rather than single period of prolonged protection. In addition, it is unclear whether repeated application of RIPC stimuli induces a state of continuous protection from IR injury. It may be possible to activate preconditioning pathways in readiness for the unpredictable nature of acute vascular events (103;375), but only if habituation does not develop. Therefore, the aims of this chapter were to determine in humans *in vivo* the time-course of protection by RIPC against endothelial IR injury, and to assess if protection against endothelial IR injury can be induced by repeated administration of RIPC stimuli over a period of 7 days. I hypothesized that, in similarity to local IPC, there are early and late phases of protection by RIPC. An additional hypothesis for this chapter was that repeated application of RIPC stimuli at regular intervals can induce continuous protection against IR injury.

4.2 Methods

4.2.1 Subjects

83 studies were performed on 23 healthy volunteers (13 men, 10 women; mean age±SD 24.3 ± 5.4 years; range, 18 to 41) who gave informed consent. Volunteer exclusion criteria are described in *section 2.1.1.2.1.* Studies were performed in a

temperature-controlled laboratory (24° to 26°C) and all studies repeated in the same volunteers were at least 7 days apart.

4.2.2 Induction of Ischaemia-Reperfusion (IR)

This was performed as described in *section 3.2.2.*

4.2.3 Induction of Remote Ischaemic Preconditioning (RIPC)

This was performed (on the arm) as described in *section 3.2.3.* The inflation/deflation cycle was performed 3 times.

4.2.4 Assessment of Conduit Vessel Function

Endothelial function of the brachial artery was assessed by flow-mediated dilatation (FMD) of the brachial artery as described in *section 2.1.1.2.*

4.2.5 Experimental Protocols

4.2.5.1 Effect of IR on endothelial function

To determine the effect of IR on endothelial function, FMD was assessed before ischaemia and at 20 minutes after reperfusion (n=13; *figure 4.1, protocol a*). This protocol causes endothelial dysfunction, but has no effect on brachial artery smooth muscle function *(section 3.3.2).*

4.2.5.2 Time-course of protection by RIPC against endothelial IR injury

FMD was assessed before and after IR preceded by RIPC (n=13; *figure 4.1, protocol b*). To determine the time-course of protection by RIPC, the RIPC stimulus was applied 4 hours (n=10; *figure 4.1, protocol c*), 24 hours (n=12; *figure 4.1, protocol d*)

and 48 hours (n=8; *figure 4.1, protocol e*) before IR. FMD was measured before and after IR.

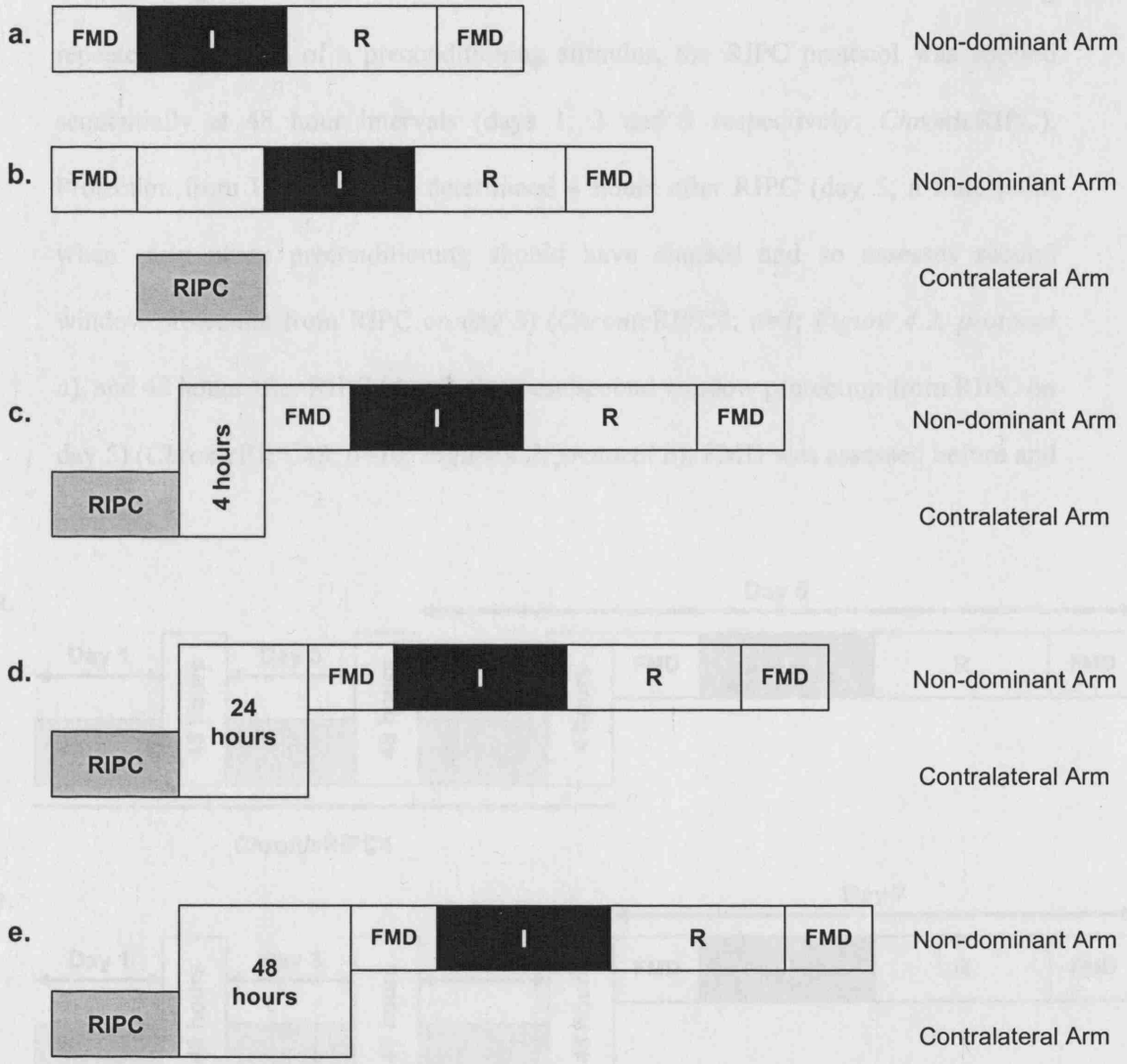


Figure 4.1 Protocol of studies to determine the time-course of protection by RIPC. FMD of the brachial artery was assessed before 20 minutes of arm ischaemia (I) and at 20 minutes of reperfusion (R) (a). The effect of RIPC of the contralateral arm on endothelial IR injury was determined by applying the RIPC stimulus immediately before IR (b). To determine the time-course of protection by RIPC, the RIPC stimulus was applied 4 hours (c), 24 hours (d) and 48 hours (e) before IR.

4.2.5.3 Sustained protection by RIPC against endothelial IR injury

10 healthy volunteers underwent assessment of the effect of IR on endothelial function *(Figure 4.1, protocol a)*. To determine whether RIPC habituates following repeated application of a preconditioning stimulus, the RIPC protocol was applied sequentially at 48 hour intervals (days 1, 3 and 5 respectively; *Chronic*RIPC). Protection from IR injury was determined 4 hours after RIPC (day 5; a time point when early phase preconditioning should have elapsed and so assesses second window protection from RIPC on day 3) (*Chronic*RIPC4; n=7; *Figure 4.2, protocol a*), and 48 hours after RIPC (day 7; to assess second window protection from RIPC on day 5) (*Chronic*RIPC48; n=10; *Figure 4.2, protocol b*). FMD was assessed before and after IR.

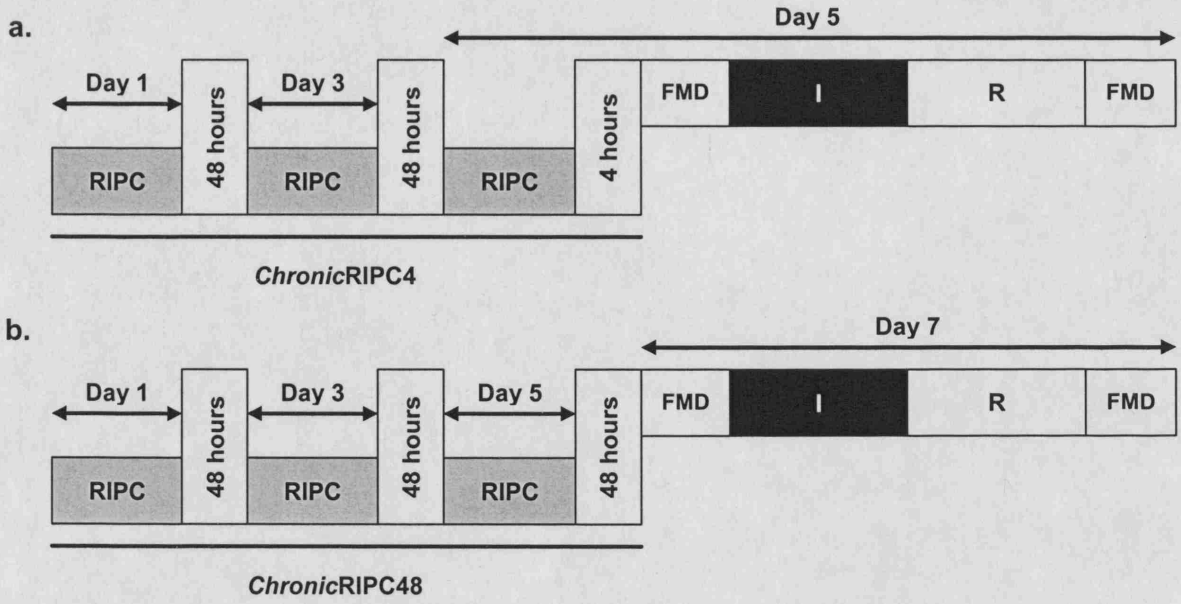


Figure 4.2 Protocol of studies to determine sustained protection by RIPC. To assess the potential of RIPC to induce continuous protection against IR (*Chronic*RIPC), RIPC was applied on the contralateral arm at 48-hour intervals (days 1, 3 and 5) followed by IR 4 hours after the application of the final RIPC stimulus on day 5 (a).

Protocol (b) was designed to determine whether repeated administration of a RIPC stimulus at 48-hour intervals over 7 days results in the development of tolerance. In the above protocols FMD was measured before and after IR.

4.2.6 Calculations and Statistics

Data expression and statistical analysis was performed as described in *section 2.3*. For multiple comparisons (5 groups for RIPC time-course, 4 groups for *Chronic*RIPC), p values by ANOVA (repeated measures) were Bonferroni adjusted. In all cases, $P<0.05$ was considered statistically significant.

4.3 Results

All subjects tolerated the procedures without any complications.

4.3.1 Effect of IR on endothelial function

The IR protocol had no effect on blood pressure and heart rate *(table 4.1, columns A and B)*. IR reduced brachial artery FMD (8.7±1.1% pre-IR versus 4.9±1.2% post-IR, $p<0.001$; n=13; *figure 4.3*) and these results could not be explained by pre- and post-IR differences in brachial artery diameter or FMD flow stimulus during reactive hyperaemia *(table 4.1, columns A and B)*.

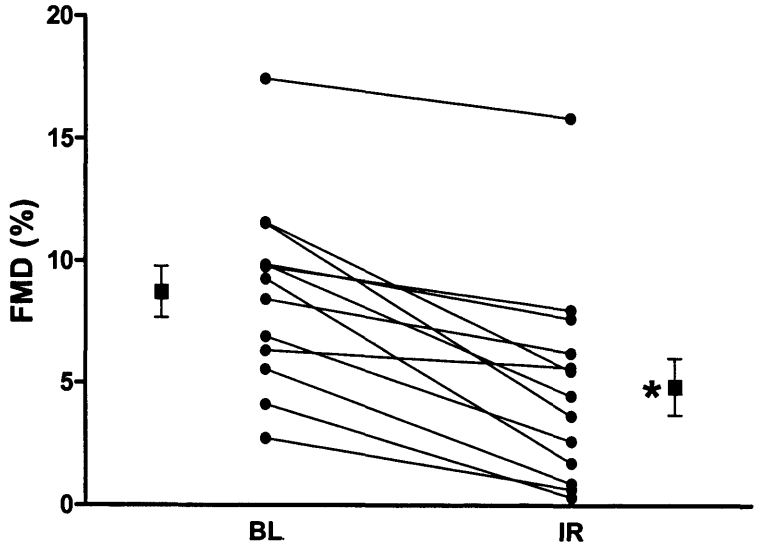


Figure 4.3 Effect of IR on endothelial function. FMD was 8.7±1.1% at baseline (BL) and was reduced by IR (a; IR 4.9±1.2%; * $p<0.001$ vs. BL, ANOVA; n=13).

4.3.3 Time-course of protection by RIPC

RIPC immediately before IR prevented endothelial dysfunction (FMD 9.4±0.7% pre- versus 8.0±0.8% post-IR+RIPC, p=NS; n=13; *figure 4.4a*) and did not alter blood pressure, heart rate, baseline arterial diameter and FMD flow stimulus *(Table 4.1, columns C and D)*. RIPC did not protect when applied 4 hours before IR (FMD 8.6±1.1% pre- versus 4.9±1.1% post-IR+RIPC4, $p<0.001$; n=10; *figure 4.4b*). When RIPC was administered 24 hours before IR, FMD was preserved (8.7±1.1% pre- versus 8.4±1.2% post-IR+RIPC24, p=NS; n=12; *figure 4.4c*). Similar findings were observed when RIPC was applied 48 hours before IR (FMD 10.0±0.9% pre- versus 8.8±1.4% post-IR+RIPC48, p=NS; n=8; *figure 4.4d*). These results could not be attributed to pre- and post-IR differences in blood pressure, heart rate, brachial artery diameter and FMD flow stimulus *(table 4.1, columns E to J)*.

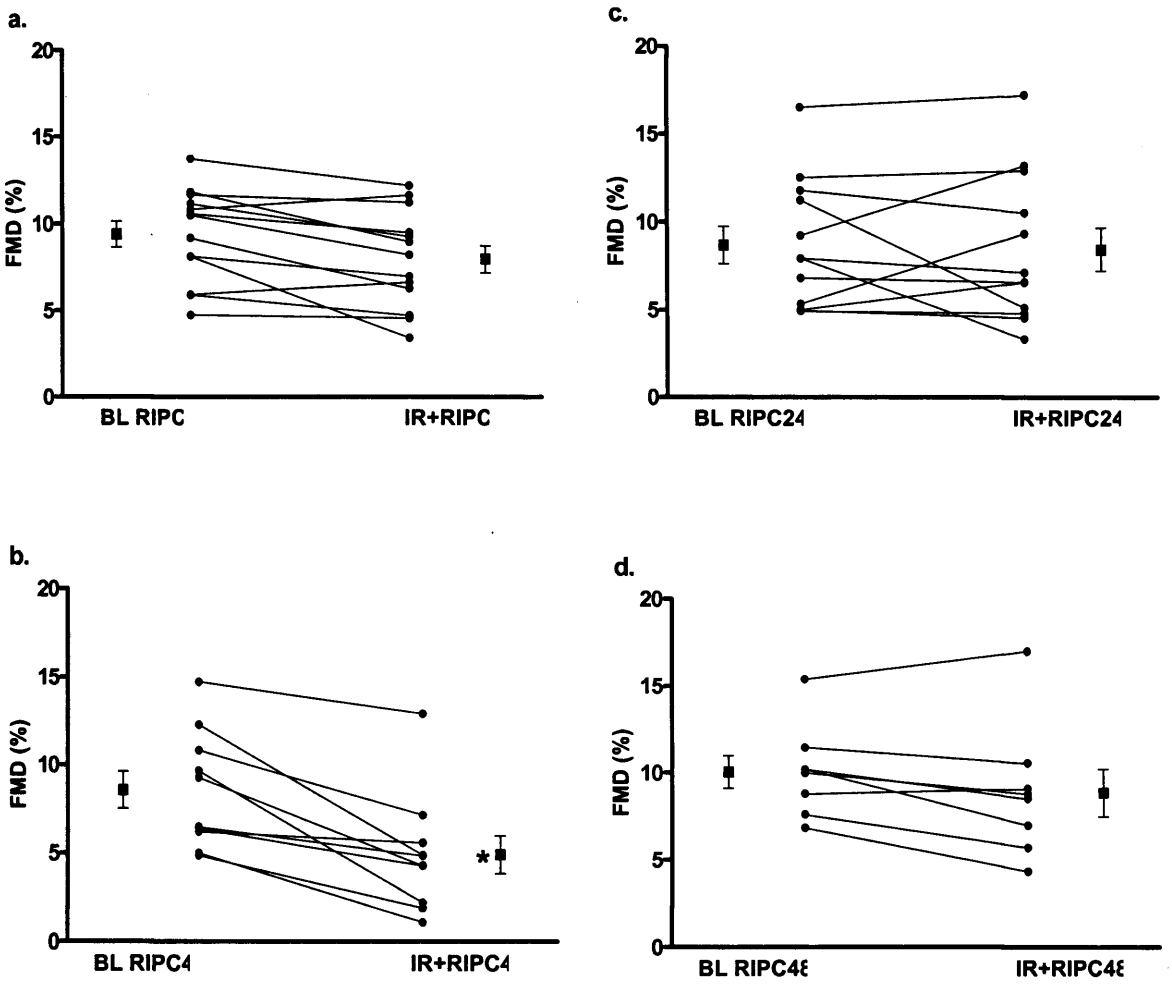


Figure 4.4 Time-course of protection by RIPC against IR-induced endothelial dysfunction. FMD was 9.4±0.7% at baseline (BL) and was unaffected by IR preceded immediately by RIPC (a; IR+RIPC 8.0±0.8%; p=NS vs. BL, ANOVA; n=13). IR reduced FMD when RIPC was applied 4 hours before IR (b; BL 8.6±1.1% vs. IR+RIPC4 4.9±1.1%; * $p<0.001$, ANOVA; n=10). However, the effect of IR to reduce FMD was prevented when RIPC was applied 24 hours before IR (c; BL 8.7±1.1% vs. IR+RIPC24 8.4±1.2%; p=NS, ANOVA; n=12) and 48 hours before (d; BL 10.0±0.9% vs. IR+RIPC48 8.4±1.4%; p=NS, ANOVA; n=8).

	IR alone (n=13)		IR+RIPC (n=13)		IR+RIPC4 (n=10)		IR+RIPC24 (n=12)		IR+RIPC48 (n=8)	
	(A) Pre	(B) Post	(C) Pre	(D) Post	(E) Pre	(F) Post	(G) Pre	(H) Post	(I) Pre	(J) Post
SBP	112±2	112±2	112±3	114±3	113±3	112±3	117±3	116±3	119±4	112±5
DBP	66±2	65±2	68±2	66±2	66±3	66±3	66±2	66±2	61±3	67±3
HR	70±3	64±3	66±2	61±3	64±3	63±3	63±2	62±2	70±6	64±3
Arterial diameter	3.8±0.2	3.8±0.2	3.8±0.2	3.8±0.2	3.7±0.2	3.6±0.2	3.8±0.2	3.7±0.2	3.6±0.2	3.5±0.3
Flow stimulus	7.4±1.0	8.6±1.6	6.5±0.7	7.0±0.9	8.8±2.5	8.5±1.7	6.9±1.0	6.7±1.6	7.0±1.4	8.0±2.0
FMD	**8.7±1.0**	**4.8±1.0***	**9.4±0.7**	**8.0±0.8**	**8.6±1.1**	**4.9±1.1*‡**	**8.7±1.0**	**8.4±1.2**	**10.0±0.9**	**8.8±1.4**

Table 4.1 Summary of pre- and post-IR data from studies designed to determine the time-course of protection by RIPC. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter was measured in millimetres and the FMD flow stimulus during reactive hyperaemia was expressed as the ratio of peak to baseline volume flow per minute (no units). FMD was expressed as peak % dilation from baseline brachial artery diameter. * *$p<0.001$ FMD (A) vs. (B), (B) vs. (D), (B) vs. (H), (E) vs. (F), (D) vs. (F) and (F) vs. (H); † $p<0.01$ FMD (F) vs. (J); ‡ $p<0.05$ FMD (B) vs. (J) (ANOVA).*

4.3.4 Sustained protection by RIPC against endothelial IR injury

IR reduced FMD (9.0±1.1% pre-IR versus 3.6±0.7% post-IR, p<0.001; n=10; *figure 4.5a*). Multiple RIPC stimuli did not alter pre- and post-IR blood pressure, heart rate, arterial diameter and flow stimulus during reactive hyperaemia *(table 4.2)*. 3 consecutive RIPC stimuli at 48-hour intervals resulted in protection when IR was induced on the non-dominant arm 48 hours following the application of the third RIPC stimulus (*Chronic*RIPC48), demonstrating that tolerance had not developed to the protective effects of RIPC over a period of 7 days (FMD 8.0±1.0% pre- versus 7.8±1.1% post-IR+*Chronic*RIPC48, p=NS; n=10; *figure 4.5c*). *Chronic*RIPC also induced protection when the non-dominant arm underwent IR 4 hours following administration of the third RIPC stimulus (*Chronic*RIPC4; FMD 9.3±1.3% pre- versus 7.6±1.0% post-IR+*Chronic*RIPC4, p=NS; n=7; *figure 4.5b*). No significant differences in baseline FMD values were observed between protocols 4.1a (IR), 4.2a (IR+*Chronic*RIPC4) and 4.2b (IR+*Chronic*RIPC*48hrs*) excluding any direct effects of multiple RIPC stimuli on brachial artery FMD *(table 4.2, columns A, C and E)*.

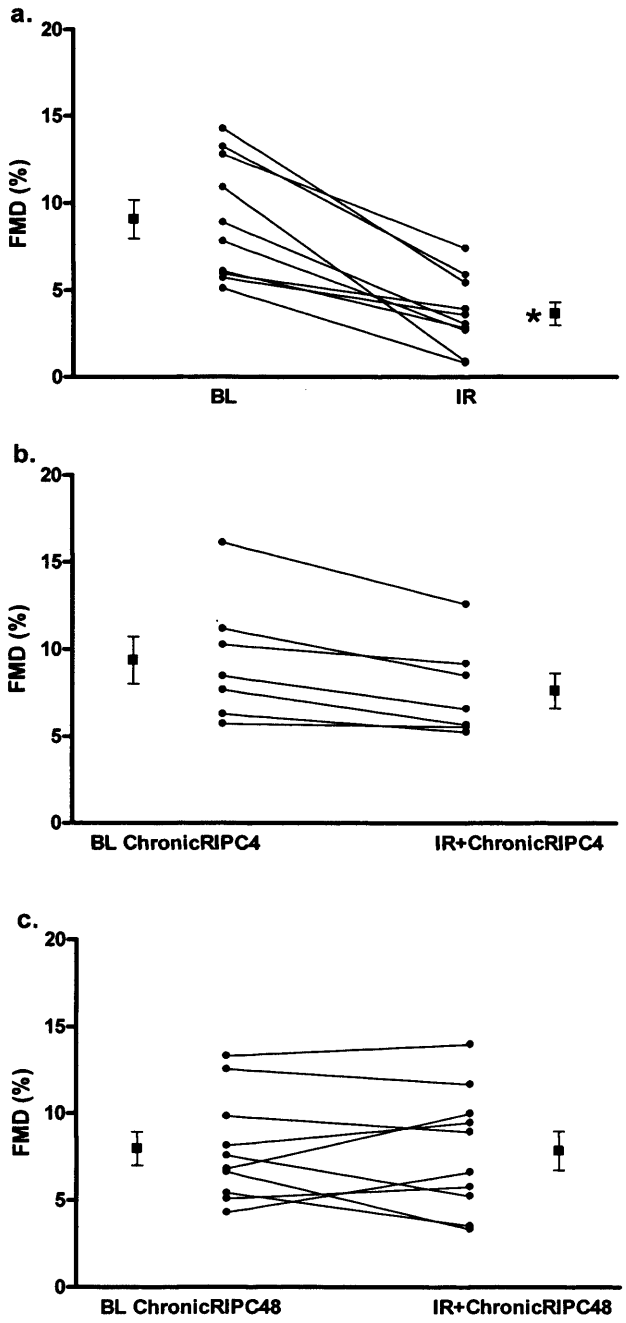


Figure 4.5 Sustained protection by RIPC against IR-induced endothelial dysfunction. IR caused a significant reduction to baseline (BL) brachial artery FMD (a; BL 9.0±1.1% vs. IR 3.6±0.7%; *p<0.001, ANOVA; n=10). Repeated application of a RIPC stimulus (3 cycles) on the arm (*Chronic*RIPC) at 48-hour intervals did not result in habituation, as *Chronic*RIPC prevented IR injury 4 hours (b; BL 9.3±1.3% vs. IR+*Chronic*RIPC4 7.6±1.0%; p=NS, ANOVA; n=7) and 48 hours after the

application of the third RIPC stimulus (c; BL 8.0±1.0% vs. IR+*Chronic*RIPC48 7.8±1.1%; p=NS, ANOVA; n=10).

	IR alone *(n=10)*		***IR+ChronicRIPC4*** *(n=7)*		***IR+ChronicRIPC48*** *(n=10)*	
	(A) Pre	*(B) Post*	*(C) Pre*	*(D) Post*	*(E) Pre*	*(F) Post*
SBP	105±3	106±3	109±8	111±9	106±2	103±2
DBP	62±2	59±4	61±5	64±9	63±2	62±2
HR	72±3	67±3	67±3	68±3	68±4	66±3
Arterial diameter	3.1±0.1	3.1±0.1	3.3±0.2	3.2±0.2	3.1±0.1	3.1±0.1
Flow stimulus	7.3±1.4	7.3±0.9	7.3±1.1	7.8±1.4	6.8±0.5	6.4±1.1
FMD	**9.0±1.1**	**3.6±0.7 *†**	**9.3±1.3**	**7.6±1.0**	**8.0±1.0**	**7.8±1.1**

Table 4.2 Summary of data from studies designed to determine the sustained protection by RIPC. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). FMD was expressed in peak % dilation from baseline diameter. * *p<0.001 FMD (A) vs. (B); † p<0.01 FMD (B) vs. (D) and (B) vs. (F).*

4.4 Discussion

This study demonstrates for the first time in humans that there are two temporally distinct phases of protection by RIPC; an early, relatively short phase, that is active immediately and disappears within 4 hours, and a second (late), more prolonged phase, that presents 24 hours after the application of the RIPC stimulus and is sustained for at least 48 hours. In addition, the protective effects against endothelial IR injury in humans can be maintained for up to 7 days, by repeated application of a RIPC stimulus, without development of tolerance to protection by RIPC.

IPC is a potent protective mechanism against IR injury in the myocardium and other tissues, but the protective effects are very transient and have been shown to disappear within 2 to 4 hours following the application of the preconditioning stimulus (72). However, a delayed form of protection reappears within 24 hours of the preconditioning stimulus, which has been referred to by Yellon's group as the "second window of protection" (SWOP) (322). Late protection by IPC, although less robust compared to immediate (early) protection, is more prolonged and has been shown to last for up to 72 hours (30;270;322). The mechanisms of early and late protection by IPC have been extensively investigated, mainly in the myocardium, and common trigger factors (538) and mediators have been identified (25;401) *(see section 1.3)*. Nonetheless, the time-frame within which the SWOP confers protection allows for the possibility of new protein synthesis, which is unlikely to be involved in the early protective phase by IPC (537). In this respect, late protection by IPC against IR injury has been associated with increased expression of heat shock proteins (105;255) and anti-oxidant enzymes (such as superoxide dysmutase) (209;270) in post-ischaemic tissues.

The results of the present study demonstrate that RIPC mirrors IPC with an early phase (first window) of protection lasting only a few hours after the RIPC stimulus, and followed 24 hours later by a second window lasting for up to 48 hours. The re-appearance of protection and its prolonged time-course is consistent with altered protein expression in the vessel wall, and it is possible that such changes may be similar to those identified in second window IPC. Further studies are needed to identify these molecular mechanisms.

The studies of the time-course of RIPC over a 7 day period indicated that there was no loss of protection with regular application of the preconditioning stimuli. Moreover, by performing RIPC repeatedly at 48 hour intervals, it was possible to eliminate the gap in preconditioning between the first and second window that follows a single application of a RIPC trigger. Although it is difficult to extrapolate beyond this time-course, it could be possible to utilise the prolonged protection offered by second window RIPC to induce a persistent or *chronic* preconditioned state (*chronic*RIPC). Animal studies have shown that repeated application of a preconditioning stimulus at regular intervals can induce continuous protection against IR injury (103;210). In contrast to local IPC, RIPC can be applied non-invasively to non-vital tissues, such as the limb (266;297), and could be administered repeatedly to induce sustained protection against IR. If the application of multiple RIPC stimuli for periods longer than 7 days does not lead to the development of tachyphylaxis to the protective effects of RIPC, such a preconditioning protocol could induce continuous protection against IR injury in the myocardium and other tissues by maintaining the second window of protection and eliminating the lack of protection observed between the early and late protective phases of RIPC (375).

4.5 Conclusions

The present study confirms that in humans RIPC offers enduring (up to 48 hours) protection against endothelial IR injury. If this is applicable to other tissues, then these observations suggest a simple way in which the effect of RIPC to reduce ischaemic damage in clinical IR syndromes can be tested. It may be possible to trigger preconditioning 24 hours in advance of cardiopulmonary bypass surgery, angioplasty or transplantation *(see chapter 8)* and provide up to 48 hours of resistance to cardiac and non-cardiac ischaemia (259;376). Moreover, the extended duration of protection by late RIPC can be exploited to achieve a state of continuous preconditioning (*chronic*RIPC) that could offer protection in high risk patients against clinical IR syndromes, in which the onset of ischaemia cannot be predicted (e.g. acute myocardial infarction or stroke).

Chapter 5:

Role of the Autonomic Nervous System in Remote Ischaemic Preconditioning

5.1 Introduction

One the most intriguing questions regarding RIPC is, how the protective signal is transferred from the site of preconditioning to remote tissues. There is evidence for humoral mediators, including endogenous opioids (117), that may reach distant tissues via the circulation *(see section 1.4.3.4.1)*. Moreover, a neurogenic pathway has been suggested as an alternative mode of transfer of protection by RIPC *(see section 1.4.3.4.2)*. Although some recent studies have looked into the role of capsaicin sensitive sensory nerves and calcitonin gene-related peptide (CGRP) (neurohumoral pathway; *see section 1.4.3.4.3*) (525), the majority of data to date demonstrate the involvement of the autonomic nervous system (ANS) in the mechanisms of RIPC (161). Nonetheless, the role of the ANS in RIPC in humans is not known at present. Thus, the aim of this study was to determine whether the early and late phases of protection by RIPC against endothelial IR injury in humans are dependent on intact autonomic function. I hypothesized that the protective signal, generated by the administration of a RIPC stimulus, is transferred by the ANS to distant tissues, resulting in early and late protection against IR injury.

5.2 Methods

5.2.1 Subjects

45 studies were performed on 8 healthy volunteers (all men; mean age±SD 27.5 ± 7.3 years; range, 21 to 41) who gave informed consent. Volunteer exclusion criteria are described in *section 2.1.1.2.1.* Studies were performed in a temperature-controlled laboratory (24° to 26°C) and all studies repeated in the same volunteers were at least 7 days apart.

5.2.2 Induction of Ischaemia-Reperfusion (IR)

This was performed as described in *section 3.2.2*

5.2.3 Induction of Remote Ischaemic Preconditioning (RIPC)

3 RIPC cycles were applied on the arm as described in *section 3.2.3*

5.2.4 Assessment of Conduit Vessel Endothelial Function

Endothelial function of the brachial artery was assessed by flow-mediated dilatation (FMD) of the brachial artery in the non-dominant arm, as described in *section 2.1.1.2.*

5.2.5 Experimental Protocols

5.2.5.1 Effect of IR on endothelial function

To determine the effect of IR on endothelial function, FMD was assessed before ischaemia and at 20 minutes after reperfusion (n=8; *figure 5.1, protocol a*).

5.2.5.2 Early and late protection by RIPC against endothelial IR injury

RIPC was applied immediately before (n=8; *figure 5.1, protocol b*) and 24 hours before (n=7; *figure 5.1, protocol* c) IR. FMD was assessed before and after IR. These studies were used as controls for subsequent early and late RIPC experiments in the presence of systemic trimetaphan *(section 5.2.5.3)*.

5.2.5.3 Role of the autonomic nervous system in early and late protection by RIPC

A venous cannula was placed in a forearm vein under local anaesthesia (1% lignocaine) and the N_N-cholinergic antagonist trimetaphan camsylate (Cambridge Laboratories, Wallsend, Tyne and Wear, UK) was infused at 1-6 mg/min, with 1mg/min dose increments at 5-minute intervals. The dose was increased until the heart rate response to a Valsalva manoeuvre was abolished. The same group of healthy volunteers were administered trimetaphan by infusion during the application of the RIPC stimulus immediately before IR (n=7; *figure 5.1, protocol d*) or 24 hours before IR (n=7; *Figure 5.1, protocol e*). The effect of trimetaphan on baseline FMD (n=4; *Figure 5.1, protocol f*) and the endothelial response to IR (n=4; Figure *5.1, protocol g*) was determined to exclude any direct effects on these measures.

5.2.6 Calculations and Statistics

Data expression and statistical analysis was performed as described in *section 2.3*. For multiple comparisons (5 groups), p values by ANOVA (repeated measures) were Bonferroni adjusted. In all cases, $P<0.05$ was considered statistically significant.

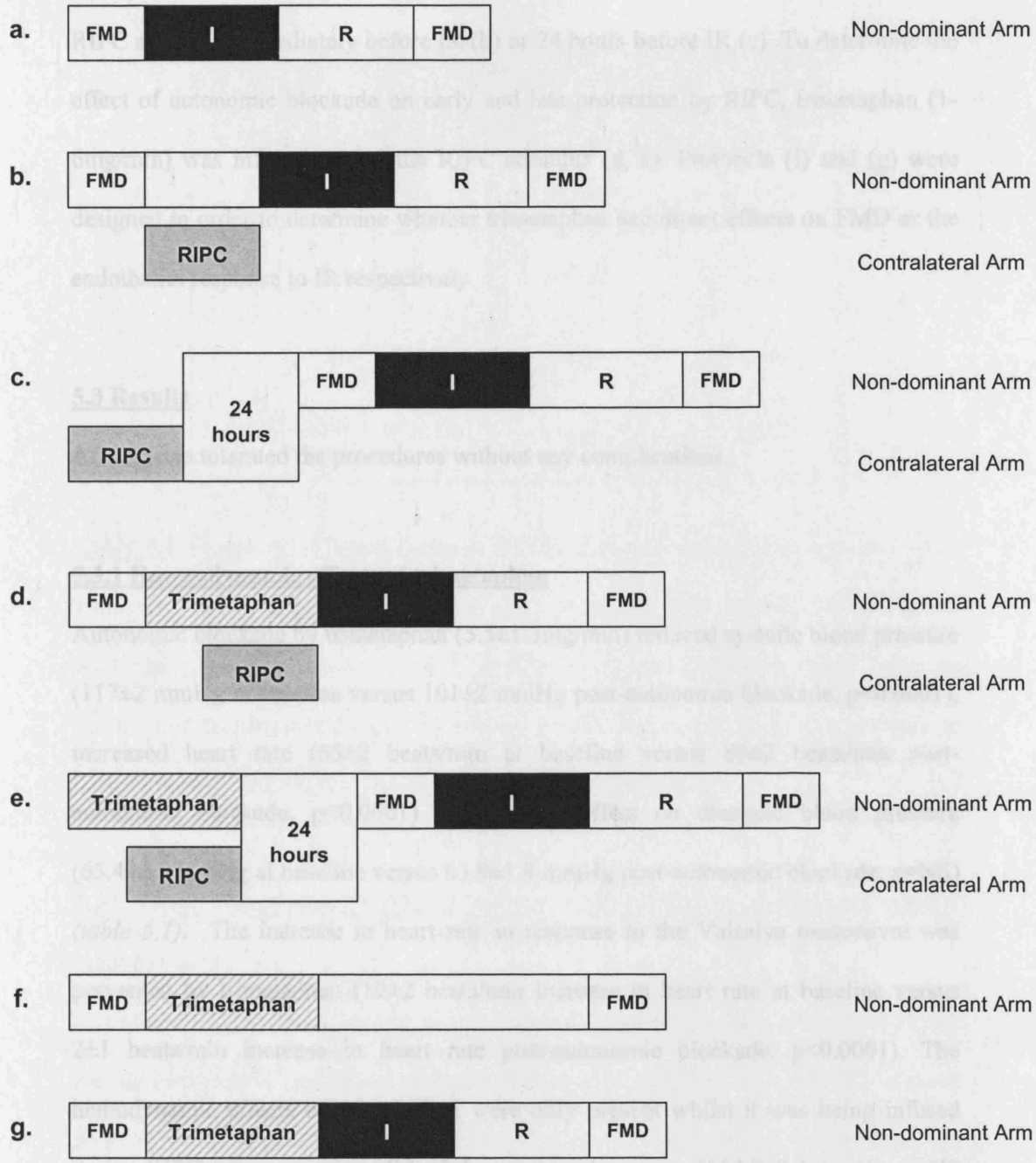


Figure 5.1 Protocol of studies to determine the effect of autonomic blockade on early and late protection by RIPC. Flow-mediated dilatation (FMD) of the brachial artery was assessed before 20 minutes of arm ischaemia (I) and at 20 minutes of reperfusion (R) (a). Early and late protective effects of remote ischaemic preconditioning (RIPC) of the contralateral arm on endothelial IR injury were determined by applying the

RIPC stimulus immediately before IR (b) or 24 hours before IR (c). To determine the effect of autonomic blockade on early and late protection by RIPC, trimetaphan (1-6mg/min) was infused during the RIPC stimulus (d, e). Protocols (f) and (g) were designed in order to determine whether trimetaphan had direct effects on FMD or the endothelial response to IR respectively.

5.3 Results

All subjects tolerated the procedures without any complications.

5.3.1 Haemodynamic effects of trimetaphan

Autonomic blockade by trimetaphan (5.5±1.3mg/min) reduced systolic blood pressure (117±2 mmHg at baseline versus 101±2 mmHg post-autonomic blockade, $p<0.0001$), increased heart rate (65±2 beats/min at baseline versus 89±2 beats/min post-autonomic blockade, $p<0.0001$) but had no effect on diastolic blood pressure (65.4±2.0 mmHg at baseline versus 63.9±1.9 mmHg post-autonomic blockade, p=NS) *(table 5.1)*. The increase in heart rate in response to the Valsalva manoeuvre was prevented by trimetaphan (19±2 beats/min increase in heart rate at baseline versus 2±1 beats/min increase in heart rate post-autonomic blockade, $p<0.0001$). The hemodynamic effects of trimetaphan were only present whilst it was being infused during RIPC; after cessation of the infusion, blood pressure (114.7±2.4 mmHg, p=NS versus baseline) and heart rate (62±2 mmHg, p=NS versus baseline) had returned to normal by the time FMD was repeated *(pooled data from 22 studies in healthy volunteers; blood pressure and heart rate values at baseline and prior to repeat assessment of endothelial function for individual trimetaphan protocols are summarised in columns C and D, tables 5.2 to 5.4).*

	ANS blockade (n=22)	
	Pre	Post
SBP	**117±2**	**101±2 §**
DBP	65±2	63±2
HR	**65±2**	**89±2 §**
Δ(HR) following Valsalva manoeuvre	**19±2**	**2±1 §**

Table 5.1 Summary of haemodynamic effects of autonomic nervous system (ANS) blockade. Data was pooled from 22 sequential studies with trimetaphan in healthy volunteers *(figure 6.1, protocols d to g)*. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute); Δ(HR): increase in heart rate in response to the Valsalva manoeuvre. *§ p<0.0001 vs. pre-ANS blockade.*

5.3.2 Effect of autonomic blockade on endothelial function and the endothelial response to IR

IR reduced FMD (7.3±1.2% pre- versus 2.6±0.7% post-IR, p<0.01; n=8; *figure 5.2a*), but did not result in significant changes in blood pressure, heart rate, arterial diameter and FMD flow stimulus *(table 5.2, columns A and B)*. Trimetaphan had no effect on baseline brachial artery FMD (6.7±1.3% pre- versus 6.8±0.9% post-trimetaphan, p=NS; n=4) and the endothelial response to IR (FMD 8.5±1.2% pre- versus 4.5±0.7% post-IR+trimetaphan, p<0.01; n=4; *figure 5.2b*). Brachial artery diameter and flow stimulus during reactive hyperaemia were not affected by trimetaphan *(table 5.2, columns C and D)*.

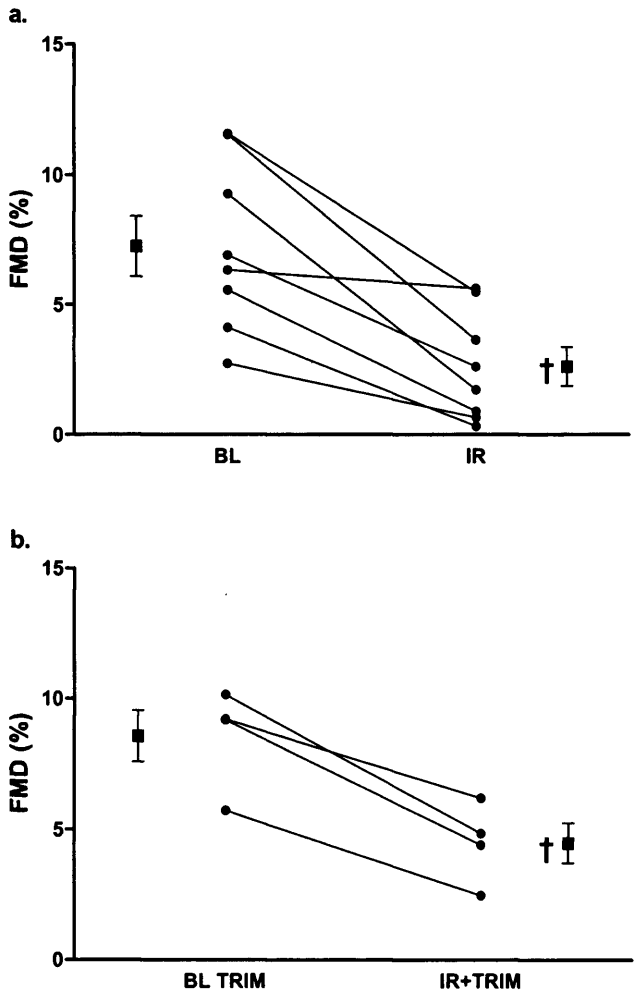


Figure 5.2 Effect of trimetaphan on endothelial response to IR. FMD was 7.3±1.2% at baseline (BL) and was reduced by IR (a; IR 2.6±0.7%; † $p<0.01$ vs. BL, ANOVA; n=8) and this was not significantly affected by trimetaphan (b; BL 8.5±1.2% vs. IR+TRIM 4.5±0.7%; † $p<0.01$, paired *t*-test; n=4).

	IR alone (n=8)		*IR+Trimetaphan (n=4)*	
	(A) Pre	*(B) Post*	*(C) Pre*	*(D) Post*
SBP	112±2	112±2	116±7	116±7
DBP	66±2	65±4	62±3	64±1
HR	70±5	64±3	64±4	61±5
Arterial diameter	4.1±0.2	4.2±0.2	3.7±0.2	3.8±0.2
Flow stimulus	6.3±1.8	8.0±1.6	8.1±2.8	7.3±2.9
FMD	**7.3±1.2**	**2.6±0.7 †**	**8.5±1.2**	**4.5±0.7 †**

Table 5.2 Summary of pre- and post-IR data from studies designed to determine the effect of trimetaphan on the endothelial response to IR. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). FMD was expressed in peak % dilation from baseline diameter. *† $p<0.01$ FMD (A) vs. (B) (ANOVA), and (C) vs. (D) (paired t-test).*

5.3.3 Effect of autonomic blockade early and late protection by RIPC

RIPC prevented endothelial dysfunction when applied immediately before IR (FMD 8.2±0.9% pre- versus 7.0±0.8% post-IR+RIPC, p=NS; n=8; *figure 5.3a*). The RIPC protocol had no effect on blood pressure, heart rate, arterial diameter and FMD flow stimulus *(table 5.3, column A and B)*. No protection was observed when RIPC was applied in the presence of systemic trimetaphan (FMD 8.3±1.1% pre- versus 4.2±0.9% post-IR+RIPC+Trimetaphan, $p<0.05$; n=7; *figure 5.3b*).

Similarly, RIPC, applied 24 hours before IR prevented IR injury (FMD 8.0±1.2% pre- versus 7.9±1.6% post-IR+RIPC24, p=NS; n=7; *figure 5.4a*), but trimetaphan blocked the protective effect of RIPC at this time-point (FMD 7.3±1.0% pre- versus 2.3±0.6% post-IR+RIPC24+Trimetaphan, $p<0.001$, n=7; *figure 5.4b*). The effect of autonomic blockade on early and late protection by RIPC could not be explained by trimetaphan-induced changes in brachial artery diameter or flow stimulus during reactive hyperaemia *(columns C and D, tables 5.3 and 5.4 respectively)*.

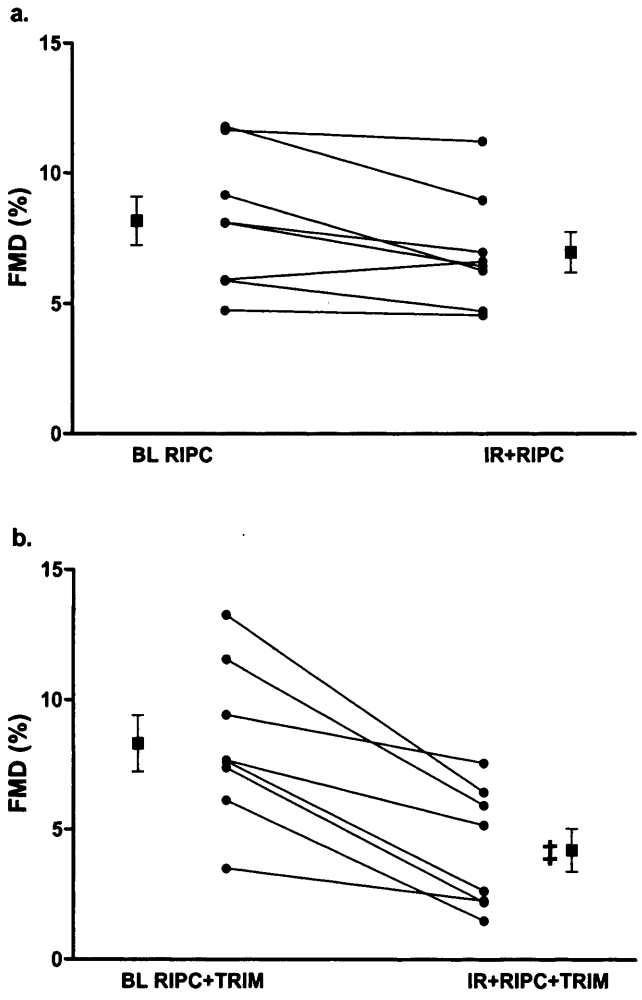


Figure 5.3 Effect of autonomic blockade on early protection by RIPC. RIPC protected against IR induced endothelial dysfunction (a; BL 8.2±0.9% vs. IR+RIPC 7.0±0.8%; p=NS, ANOVA; n=8) but the protective effects disappeared when the RIPC stimulus was administered in the presence of systemic trimetaphan (b; BL 8.3±1.1% vs. IR+RIPC+TRIM 4.2±0.9%; ‡ $p<0.05$, ANOVA; n=7).

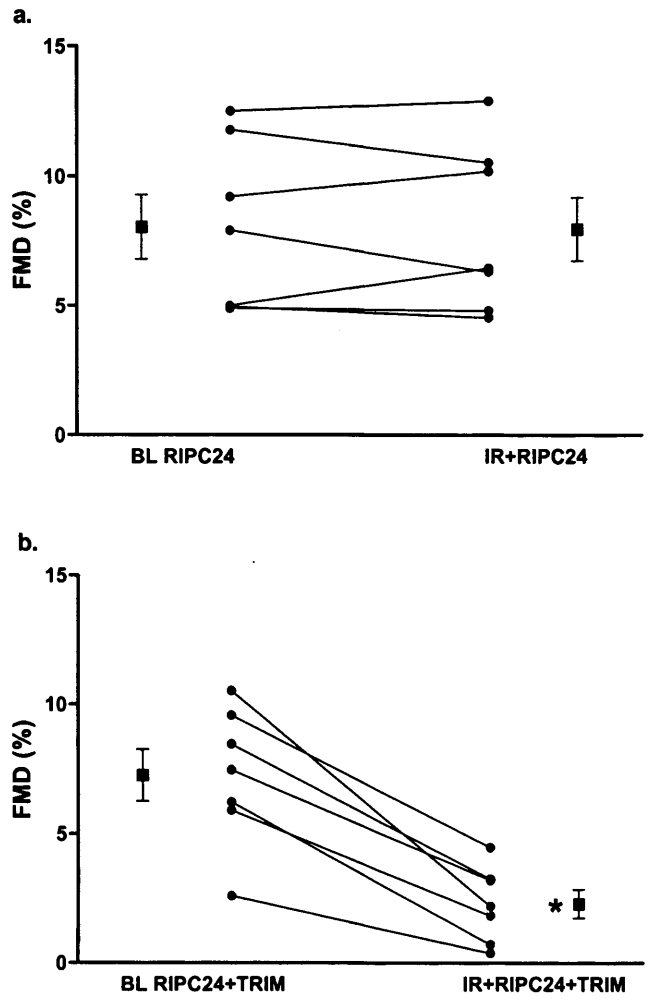


Figure 5.4 Effect of autonomic blockade on late protection by RIPC. RIPC prevented IR-induced endothelial dysfunction when applied 24 hours in advance of IR (a; BL 8.0±1.2% vs. IR+RIPC24 7.9±1.2%; p=NS, ANOVA; n=7). Late protection by RIPC was blocked when trimetaphan was administered at the same time as RIPC (b; BL 7.3±1.0% vs. IR+RIPC24+TRIM 2.3±0.6%; *p<0.001, ANOVA; n=7).

	IR+RIPC (n=8)		IR+RIPC+Trimetaphan (n=7)	
	(A) Pre	(B) Post	(C) Pre	(D) Post
SBP	115±2	117±3	117±3	118±3
DBP	65±2	67±2	60±3	63±2
HR	64±4	63±3	67±3	64±3
Arterial diameter	4.0±0.2	4.0±0.2	3.8±0.2	3.8±0.2
Flow stimulus	5.4±2.0	5.3±1.9	6.4±1.7	8.6±1.1
FMD	**8.2±0.9**	**7.0±0.8 †**	**8.3±1.1**	**4.2±0.9 ‡**

Table 5.3 Summary of data from studies designed to determine the effect of autonomic blockade on early protection by RIPC. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). FMD was expressed in peak % dilation from baseline diameter. *‡ $p<0.05$ FMD (C) vs. (D) and (B) vs. (D); † $p<0.01$ (B) vs. post-IR alone (Table 6.2, column B) (ANOVA).*

	IR+RIPC24 (*n=7*)		IR+RIPC24+Trimetaphan (*n=7*)	
	(A) Pre	*(B) Post*	*(C) Pre*	*(D) Post*
SBP	113±2	114±3	112±4	113±5
DBP	65±2	67±3	60±3	63±2
HR	62±4	61±4	64±4	63±4
Arterial diameter	4.1±0.2	4.1±0.2	4.1±0.3	4.2±0.3
Flow stimulus	5.3±1.4	6.3±1.6	5.6±0.7	7.3±1.9
FMD	**8.0±1.2**	**7.9±1.6** *	**7.3±1.0**	**2.3±0.6** *

Table 5.4 Summary of data from studies designed to determine the effect of autonomic blockade on late protection by RIPC. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). FMD was expressed in peak % dilation from baseline diameter. **p<0.001 FMD (C) vs. (D), (B) vs. (D) and (B) vs. post-IR alone (Table 6.2, column B) (ANOVA).*

5.4 Discussion

This study establishes for the first time in humans that protection by RIPC is dependent on intact autonomic function. The autonomic nervous system (ANS) is involved in the mechanisms of both early and late protective phases of RIPC.

In the present studies in healthy volunteers, the autonomic ganglion blocker trimetaphan was administered by intravenous infusion at a dose sufficient to cause autonomic block (confirmed by its effects on blood pressure, heart rate and the Valsalva response). Because of its short-lived action, autonomic blockade was restricted to the RIPC phase of the protocols, and baseline hemodynamics were restored in advance of the repeat assessment of endothelial function. Time-control studies confirmed that trimetaphan had no direct effect on FMD, or the endothelial response to IR injury. However, when administered during RIPC, it blocked its early and late protective effects against endothelial IR injury. It is possible that release of local triggers of IPC (including bradykinin and adenosine) activate the autonomic nervous system either directly (306;423), or via sensory nerves (213;471;525), and transfer the signal to remote sites. How this leads to tissue protection is not clear at present, but it may involve ANS-mediated activation of protein kinase C in distant tissues (520). Activation of PKC is a key event in the signal transduction pathway of both local and remote IPC, and may induce protection via K_{ATP} channel opening or other unidentified end-effectors *(see section 1.4.3.2.1)*.

The experimental evidence presented in this chapter does not indicate which component of autonomic function (muscarinic or adrenergic) is involved, and further studies are required to dissect these pathways. Another potential limitation is the lack

of specificity of trimetaphan which, in addition to its ability to inhibit autonomic function (254), also has α-adrenoreceptor blocking properties (190) and induces the release of histamine (139). Moreover, trimetaphan has direct vasodilator actions, although the mechanisms of this effect are not currently known (190). Although these additional actions are unlikely to account for the effects described in the present study, unknown effects of the drug that alter the response of the vascular endothelium to RIPC cannot be excluded. One way of eliminating this potential source of error is to test whether RIPC can be induced in patients with ANS dysfunction (438).

5.5 Conclusions

This is the first demonstration in humans *in vivo* that the autonomic nervous system is involved in the mechanisms of spread of protection by RIPC. Despite remaining uncertainties regarding the signal transduction pathway through which ANS activation results in early and late protection of remote tissues against IR, the present results are consistent with a key role for autonomic activation in this process. However, these findings do not exclude the involvement of humoral mediators in RIPC, and it is possible that the protective effects are mediated by both neuronal and humoral pathways acting synergistically.

Chapter 6:

Role of ATP-sensitive Potassium Channels in Remote Ischaemic Preconditioning

6.1 Introduction

Opening of ATP-sensitive potassium (K_{ATP}) channels has been consistently demonstrated in the mechanisms of protection by preconditioning in various animal and human models of IR injury (175;538). K_{ATP} channels have also been implicated as triggers, mediators and end effectors in the signal transduction pathway of RIPC *(sections 1.4.3.2.3 and 1.4.3.3.2)*. However, the role of K_{ATP} channels in RIPC in humans is not currently known. Therefore, the hypothesis for this study was that protection by RIPC against endothelial IR injury in humans is dependent on opening of K_{ATP} channels. The non-specific K_{ATP} channel blocker glibenclamide was used in order to investigate the role of K_{ATP} channels in the mechanisms of RIPC.

6.2 Methods

6.2.1 Subjects

40 studies were performed on 10 healthy volunteers (all men; mean age±SD 22.9 ± 6.6 years; range, 18 to 41) who gave informed consent. Volunteer exclusion criteria are described in *section 2.1.1.2.1*. Studies were performed in a temperature-controlled laboratory (24° to 26°C) and all studies repeated in the same volunteers were at least 7 days apart.

6.2.2 Induction of Ischaemia-Reperfusion (IR)

IR was induced on the non-dominant arm as previously described *(section3.2.2)*.

6.2.3 Induction of Remote Ischaemic Preconditioning (RIPC)

3-cycle RIPC was induced on the contralateral arm as described in *section 3.2.3*.

6.2.4 Assessment of Conduit Vessel Endothelial Function

Endothelial function of the brachial artery was assessed by flow-mediated dilatation (FMD) of the brachial artery in the non-dominant arm, as described in *section 2.1.1.2.*

6.2.5 Experimental Protocols

6.2.5.1 Effect of IR on endothelial function

To determine the effect of IR on endothelial function, FMD was assessed before ischaemia and at 20 minutes after reperfusion (n=10; *figure 6.1, protocol a*).

6.2.5.2 Protection by RIPC against endothelial IR injury

To determine the protective effects of RIPC on IR injury, FMD was assessed before and after IR immediately preceded by RIPC (n=10; *figure 6.1, protocol* b). These studies were used as controls for subsequent RIPC experiments on the same volunteers in the presence of systemic glibenclamide *(section 6.2.5.3).*

6.2.5.3 Role of K_{ATP} channels in protection by RIPC

To assess the role of K_{ATP} channels in protection by RIPC, a 5mg dose of the non-selective K_{ATP} channel blocker glibenclamide was administered orally, in the same group of healthy volunteers. To achieve peak blood concentration of the drug before the start of the study, glibenclamide was administered 45 minutes prior to baseline FMD assessment (360). This was followed by the application of the RIPC stimulus on the contralateral arm, IR and a final FMD assessment, as described above (n=10; *figure 6.1 protocol c*). To exclude a direct effect of glibenclamide on endothelial IR injury, the protocol was repeated without RIPC (n=10; *figure 6.1 protocol d*). In all studies, a high-carbohydrate meal (340kcal) was given immediately and 3 hours after

(750kcal) the administration of glibenclamide (416). Blood glucose levels were monitored throughout the study.

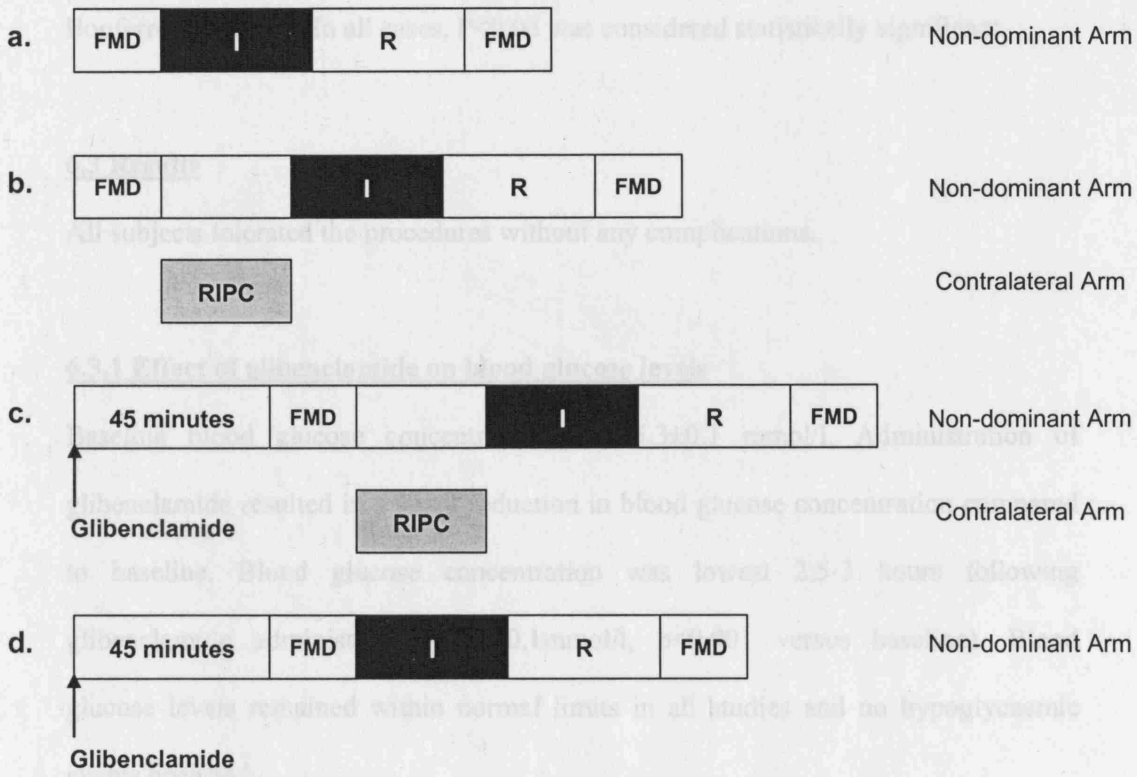


Figure 6.1 Protocol of studies to determine the effect of K_{ATP} channel blockade on early and late protection by RIPC. Flow-mediated dilatation (FMD) of the brachial artery was assessed before 20 minutes of arm ischaemia (I) and at 20 minutes of reperfusion (R) (a). The protective effects of remote ischaemic preconditioning (RIPC) of the contralateral arm on endothelial IR injury were determined by applying the RIPC stimulus immediately before IR (b). To determine the role of K_{ATP} channels in protection by RIPC, protocol (b) was repeated with glibenclamide (5mg) administered 45 minutes before baseline endothelial function assessment (c). Protocol (d) was designed to exclude any direct effects of glibenclamide on the endothelial response to IR.

6.2.6 Calculations and Statistics

Data expression and statistical analysis was performed as described in *section 2.3*. For multiple comparisons (4 groups), p values by ANOVA (repeated measures) were Bonferroni adjusted. In all cases, $P<0.05$ was considered statistically significant.

6.3 Results

All subjects tolerated the procedures without any complications.

6.3.1 Effect of glibenclamide on blood glucose levels

Baseline blood glucose concentration was 5.3±0.1 mmol/l. Administration of glibenclamide resulted in a small reduction in blood glucose concentration compared to baseline. Blood glucose concentration was lowest 2.5-3 hours following glibenclamide administration (4.2±0.1mmol/l, $p<0.001$ versus baseline). Blood glucose levels remained within normal limits in all studies and no hypoglycaemic events occurred.

6.3.2 Effect of K_{ATP} channel blockade on endothelial function and the endothelial response to IR

IR resulted in brachial artery endothelial dysfunction (FMD 7.4±0.7% pre- versus 2.7±0.4% post-IR, $p<0.001$; n=10; *figure 6.2a*), but did not cause significant changes in blood pressure, heart rate, arterial diameter and FMD flow stimulus *(table 6.1, columns A and B)*. Glibenclamide did not affect the endothelial response to IR (FMD 7.6±0.7% pre- versus 2.6±0.4% post-IR+glibenclamide, $p<0.001$; n=10; *figure 6.2b*) and had no effect on baseline brachial artery FMD *(table 6.1 columns A and C)*.

Baseline blood pressure, heart rate, arterial diameter and flow stimulus during reactive hyperaemia were also not affected by glibenclamide *(table 6.1 columns A and C)*.

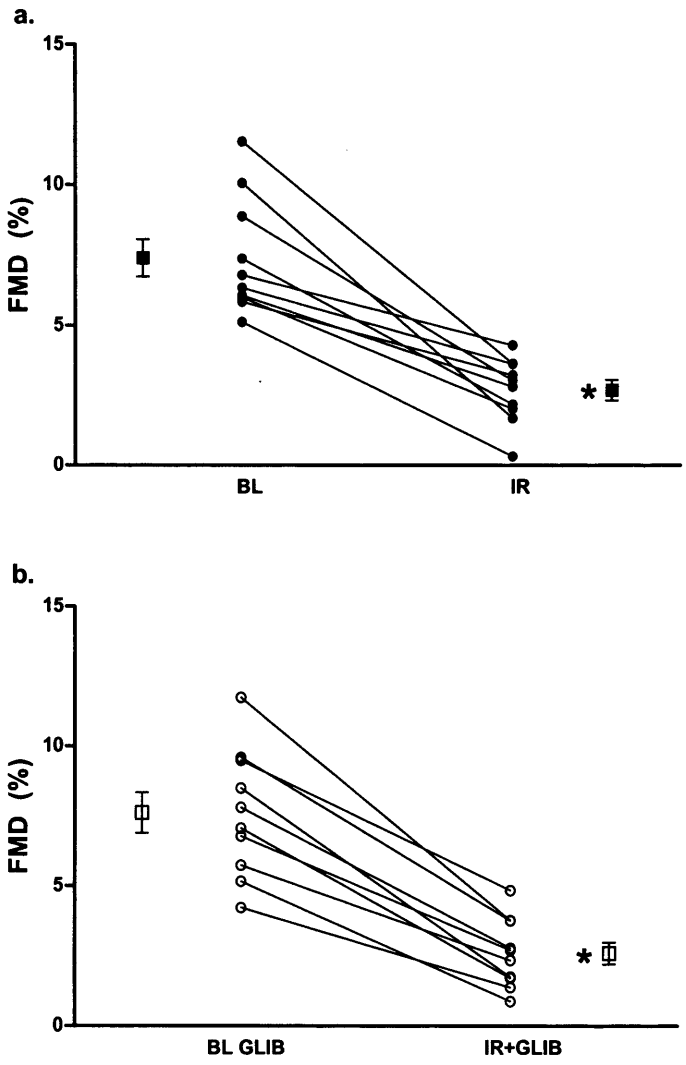


Figure 6.2 Effect of glibenclamide on endothelial response to IR. IR resulted in a significant reduction in brachial artery FMD compared to baseline (BL) (a; BL 7.4±0.7% vs. IR 2.7±0.4%; *$p<0.001$, ANOVA; n=10) and this was not affected by glibenclamide (b; BL 7.6±0.7% vs. IR+GLIB 2.6±0.4%; *$p<0.001$, ANOVA; n=10).

	IR alone (n=10)		IR+ Glibenclamide (n=10)	
	(A) Pre	(B) Post	(C) Pre	(D) Post
SBP	111±3	109±3	112±3	113±2
DBP	60±2	64±3	59±3	61±2
HR	65±3	60±3	65±4	64±4
Arterial diameter	3.5±0.1	3.4±0.1	3.4±0.1	3.5±0.1
Flow stimulus	8.7±1.6	10.4±1.9	9.4±1.3	8.2±1.6
FMD	**7.4±0.7**	**2.7±0.4** *	**7.6±0.7**	**2.6±0.4** *

Table 6.1 Summary of pre- and post-IR data from studies designed to determine the effect of glibenclamide on the endothelial response to IR. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). FMD was expressed in peak % dilation from baseline diameter. * *$p<0.001$ FMD (A) vs. (B) and (C) vs. (D) (ANOVA).*

6.3.3 Effect of K_{ATP} channel blockade on protection by RIPC

RIPC prevented endothelial dysfunction when applied immediately before IR (FMD 7.8±0.7% pre- versus 6.6±0.7% post-IR+RIPC, p=NS; n=10; *figure 6.3a*). These results could not be explained by RIPC-induced changes in blood pressure, heart rate, brachial artery diameter and flow stimulus during reactive hyperaemia *(table 6.2, columns A and B)*. In contrast, RIPC had no effect against endothelial IR injury in the presence of glibenclamide (FMD 7.1±0.9% pre- versus 3.2±0.5% post-IR+RIPC+GLIB, p<0.001; n=10; *figure 6.3b*). This could not be attributed to direct effects of glibenclamide on blood pressure, heart rate, arterial diameter and FMD flow stimulus *(table 6.2)*.

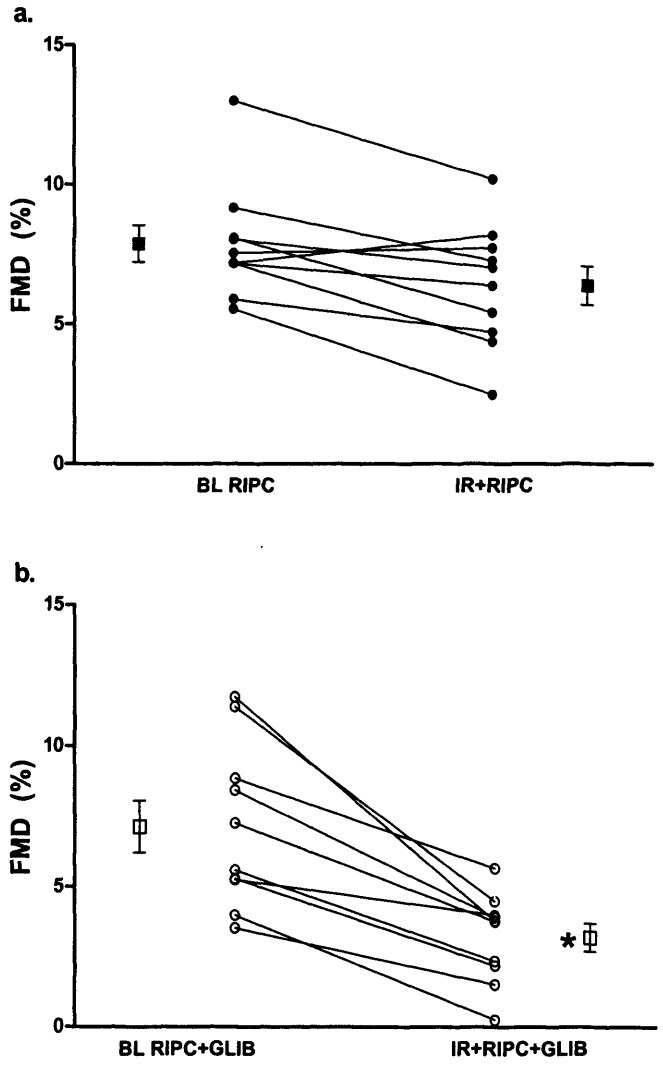


Figure 6.3 Effect of K_{ATP} channel blockade on protection by RIPC. RIPC protected against endothelial IR injury (a; BL 7.8±0.7% vs. IR+RIPC*Arm3C* 6.6±0.7%; p=NS, ANOVA; n=10). The protective effects of RIPC were abrogated by systemic glibenclamide (5mg) administered 45 minutes before baseline FMD (d; BL 7.1±0.9% vs. IR+RIPC+GLIB 3.2±0.5%; *p<0.001, ANOVA; n=10).

	IR+RIPC (n=10)		IR+ RIPC+Glibenclamide (n=10)	
	(A) Pre	(B) Post	(C) Pre	(D) Post
SBP	116±4	116±4	111±3	114±4
DBP	63±2	65±2	60±3	61±1
HR	66±4	62±3	66±3	68±5
Arterial diameter	3.4±0.1	3.4±0.2	3.4±0.1	3.4±0.1
Flow stimulus	11.2±1.1	9.9±1.7	9.1±1.2	10.0±0.9
FMD	**7.8±0.7**	**6.6±0.7 §**	**7.1±0.9**	**3.2±0.5 ***

Table 6.2 Summary of pre- and post-IR data from studies designed to determine the effect of K_{ATP} channel blockade by glibenclamide on protection by RIPC. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). FMD was expressed in peak % dilation from baseline diameter. * *$p<0.001$ FMD (C) vs. (D) and (B) vs. (D); § $p<0.001$ FMD vs. post-IR alone (table 6.1, column B) (ANOVA).*

6.4 Discussion

This study suggests, for the first time in humans, that K_{ATP} channels are involved in the mechanisms of protection by RIPC against endothelial IR injury of conduit vessels.

Glibenclamide was used to provide systemic blockade of K_{ATP} channels during induction of RIPC. Glibenclamide abolished protection induced by RIPC, which is in agreement with evidence from animal studies (120;259;262;342). The present findings are also consistent with previous observations on the action of glibenclamide to block local IPC in the forearm resistance bed in humans (67), demonstrating further similarities between "classic" and remote preconditioning and supporting the notion that IPC and RIPC are facets of the same biological phenomenon. In the present investigation, glibenclamide was administered at a dose similar to that used in clinical practice (3). K_{ATP} channel blockade did not affect baseline FMD and did not exacerbate IR injury. Thus, the effect of glibenclamide on RIPC could not be attributed to direct actions of glibenclamide to alter baseline endothelial function or the endothelial response to IR. Whilst it did not exacerbate IR injury, treatment with glibenclamide at clinical doses is likely to block local or remote preconditioning when applied in the experimental or clinical setting (138;409). Indeed, glibenclamide has been shown to prevent protection by local preconditioning in patients undergoing coronary angioplasty (480).

Glibenclamide is a non-selective K_{ATP} channel blocker, and as a result the current study did not determine the exact cellular location of the channels involved in protection by RIPC. In the case of local IPC, there is evidence for involvement of both sarcolemmal and mitochondrial K_{ATP} channels in the mechanisms of protection,

based on the actions of known pharmacological modulators of channel activity (174). However, most investigators agree on a pivotal role for mitochondrial K_{ATP} channels in the phenomenon (157;311). All studies to date on the role of K_{ATP} channels opening in protection by RIPC have demonstrated that the mitochondrial subtype is involved, as specific inhibitors of sarcolemmal K_{ATP} channels failed to block the protective effects of RIPC (262;342). Moreover, previous studies on healthy volunteers using a similar model of IR on the arm, demonstrated that the specific K_{ATP} channel opener diazoxide could protect against endothelial injury in resistance vessels, and the protective effects could be abolished by glibenclamide (67). Thus, it is possible that mitochondrial K_{ATP} channels mediate the protection by RIPC observed in the present study, although further studies are required to confirm this.

How activation of K_{ATP} channels results in protection by IPC and RIPC is not clear at present, although animal studies have implicated K_{ATP} channels in both the trigger and mediator/end-effector limbs of the mechanism *(sections 1.4.3.2.3 and 1.4.3.3.2)*. In the current study, glibenclamide was administered 45 minutes prior to RIPC induction, in order to achieve peak concentrations during the application of the stimulus. However, glibenclamide has a half-life of 10 hours (402) and it is therefore likely that the drug was present in adequate amounts to result in K_{ATP} channel blockade not only during RIPC, but also during IR. As a result, it was not possible to draw conclusions as to whether K_{ATP} channel opening triggers or mediates protection by RIPC. Although a dual role for K_{ATP} channels cannot be excluded (538), studies, in which glibenclamide is administered following induction of RIPC, prior to IR, would provide valuable information regarding the position of these channels in the signal transduction pathway of RIPC in humans.

6.5 Conclusions

The present study demonstrates for the first time that RIPC of human blood vessels *in vivo* involves activation of K_{ATP} channels. Although it is not certain how K_{ATP} channel activation induces protection against IR, it is clear that opening of these channels is a key step in the mechanisms of protection by RIPC. At a clinically relevant dose, glibenclamide abolished the protective effects of RIPC, which may have implications for the design of cardioprotective strategies in patients with diabetes. Preservation of K_{ATP} channel activity may be required in order for such strategies to be beneficial against IR injury in the myocardium and other tissues.

Chapter 7:

Effect of Remote Ischaemic Preconditioning on Reperfusion Injury

7.1 Introduction

Despite ischaemic preconditioning (IPC) being the focus of extensive research over the last two decades, the protective potential of IPC has not been realized clinically (53;253). In part this is because the preconditioning stimulus needs to be applied prior to the onset of index ischaemia, which is difficult to predict in the most common clinical IR syndromes, such as acute myocardial infraction and stroke. However, emerging evidence suggests that much of the tissue damage that during IR is sustained during early reperfusion rather than during ischaemia *(see section 1.2.2.2)*. In this respect, several pharmacological agents have been shown to reduce IR injury when administered during the initial stages of reperfusion (38;219;351;529). Moreover, beneficial effects of modifying the conditions of reperfusion (e.g. gradual reperfusion) have been long known (364;418) and led to the introduction of a novel strategy targeting the reperfusion phase of IR, termed ischaemic post-conditioning (PostC) (546). This is a phenomenon whereby brief intermittent episodes of ischaemia and reperfusion applied at the onset of reperfusion reduce IR injury, and has been shown to be protective in several animal models of myocardial infarction (503). PostC was reported to be as effective as IPC in limiting IR injury (186), and since its initial description by Vinten-Johansen's group (546), it has generated renewed interest in the reperfusion phase of IR as a target for protection (487).

It is currently not known whether preconditioning offers protection by modifying the ischaemic or reperfusion phase of IR. For "classic" (local) IPC, it is not possible to discriminate between tissue protection during ischaemia or reperfusion, as preconditioning is induced in advance of index ischaemia and so may modify either phase of injury. In contrast, the preconditioning stimulus of RIPC is applied at a site

remote from that subjected to IR, and can therefore be initiated during index ischaemia. If early reperfusion is a critical phase of endothelial injury in humans, then it should be prevented by PostC, and RIPC should provide similar protection when it is initiated during index ischaemia. Such "real time" protection by RIPC would suggest that RIPC has potential to induce tissue protection in the acute phase of an ischaemic event. This chapter aimed to determine whether the application of an RIPC stimulus during index ischemia protected from IR injury to the endothelium. A novel protocol to investigate the protective effects of PostC was developed so that protection from RIPC could be compared to PostC. I hypothesized that RIPC, administered during ischaemia, and PostC, applied at the onset of reperfusion, would offer protection against endothelial IR injury in humans by modifying events occurring at the early stages of the reperfusion phase of IR.

7.2 Methods

7.2.1 Subjects

68 studies were performed on 21 healthy volunteers (13 men, 10 women; mean age±SD 24.1 ± 6.3 years; range, 18 to 48) who gave informed consent. Volunteer exclusion criteria are described in *section 2.1.1.2.1.* Studies were performed in a temperature-controlled laboratory (24° to 26°C) and all studies repeated in the same volunteers were at least 7 days apart.

7.2.2 Induction of Ischaemia-Reperfusion (IR)

The non-dominant arm was made ischaemic as described in *section 3.2.2.*

7.2.3 Induction of Remote Ischaemic Preconditioning (RIPC)

Remote ischaemic preconditioning (RIPC) was induced on *(i)* the *contralateral* arm, or *(ii)* the *contralateral* leg, as previously described *(section 3.2.3)*.

7.2.4 Induction of Post-conditioning (PostC)

Post-conditioning (PostC) was induced by applying intermittent short periods of ischaemia and reperfusion on the *non-dominant* arm early during reperfusion. Following index ischaemia, the arm was allowed to reperfuse for 10 seconds, after which the blood pressure cuff was inflated again to 200 mmHg, making the arm ischaemic for another 10 seconds. This deflation/inflation cycle was repeated for a total of 3 times (1 minute total).

7.2.5 Assessment of Conduit Vessel Function

Endothelial function of the brachial artery was assessed by flow-mediated dilatation (FMD) of the brachial artery in the non-dominant arm, as described in *section 2.1.1.2*.

7.2.6 Experimental protocols

7.2.6.1 Effect of RIPC on the reperfusion phase of IR

10 healthy volunteers underwent assessment of the effects of IR on endothelial function *(Figure 7.1, protocol a)*. This protocol has been shown to result in endothelial dysfunction but has no effect in vascular smooth muscle function *(section 3.3.2)*. To determine whether RIPC can induce protection by modifying the reperfusion phase of endothelial IR injury (*Acute*RIPC), 2 RIPC cycles where applied on the contralateral leg during the period of ischaemia on the non-dominant arm (*Acute*RIPC*Leg2C*; n=8; *figure 7.1, protocol b*). This stimulus allowed the completion

of the preconditioning protocol during the 20-minute ischaemic insult (index ischaemia) to the non-dominant arm. I have shown in *chapter 3* that this protocol induces protection against IR when applied in advance of index ischaemia *(section 3.3.3.2)*. To determine whether it was necessary for the RIPC stimulus to be applied in full during ischaemia, a 3-cycle RIPC stimulus was administered on the contralateral arm during ischaemia and early reperfusion (*Acute*RIPC*Arm3C*; n=10; Figure 2, protocol e). As can be seen in *figure 7.1*, this protocol allows for the completion of 2 cycles of RIPC on the arm during index ischaemia (which does not prevent endothelial dysfunction during IR when applied in advance of index ischaemia; *see section 3.3.3.1*). The final cycle of RIPC in this protocol is completed after reperfusion has begun.

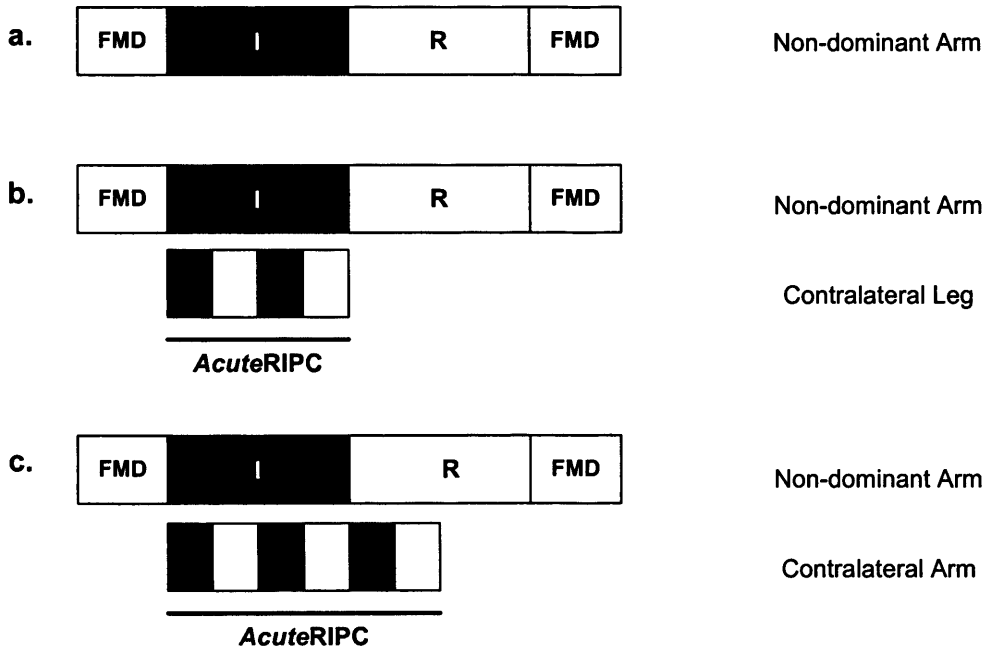


Figure 7.1 Protocol of studies to determine acute protective effects of RIPC. Flow-mediated dilatation (FMD) of the brachial artery was assessed before 20 minutes of arm ischaemia (I) and at 20 minutes of reperfusion (R) (a). The effects of RIPC on the reperfusion phase of IR injury were determined by applying 2 RIPC cycles on the

contralateral leg during index ischaemia (b). To assess whether the RIPC stimulus needs to be applied in full during ischaemia, 3 RIPC cycles were applied on the contralateral arm during ischaemia and early reperfusion (c).

7.2.6.2 Effect of PostC on endothelial IR injury

The effects of IR on endothelial function were assessed in a group of 11 healthy volunteers *(figure 7.1, protocol a)*. In order to establish that protection against endothelial IR can be achieved by modifying reperfusion, PostC was induced by applying 3 cycles of 10 seconds reperfusion and 10 seconds ischaemia (PostC) *immediately* following index ischaemia (n=11; *figure 7.2, protocol a*). To determine whether PostC needs to be applied at the onset of reperfusion, a 1-minute period of arm reperfusion was allowed prior to the application of the PostC stimulus (*Delayed*PostC; n=7; Figure 7.2, protocol b).

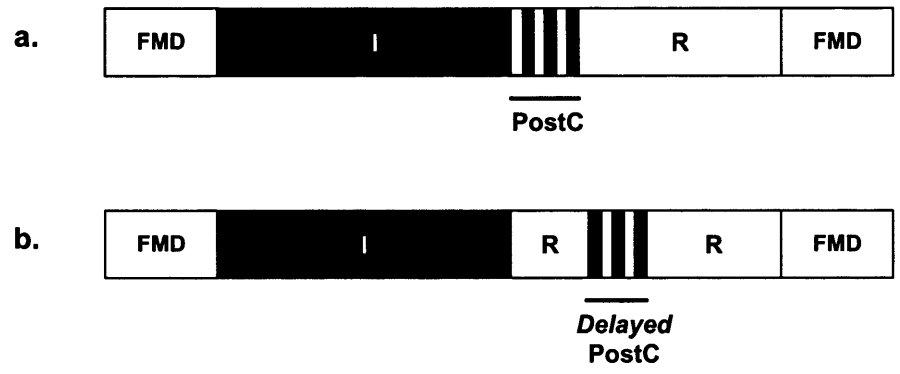


Figure 7.2 Protocols of studies to determine the effects of post-conditioning (PostC) on IR injury. To determine whether modification of reperfusion by PostC can result in protection against endothelial IR injury, 3 cycles of 10 seconds arm reperfusion and 10 seconds ischaemia were applied immediately following restoration of blood supply to the arm (a). In order to establish if there is a requirement for PostC to be applied at

the onset of reperfusion, the PostC stimulus was administered following 1 minute of arm reperfusion (*Delayed*PostC; b).

7.2.7 Calculations and Statistics

Data expression and statistical analysis was performed as described in *section 2.3*. For multiple comparisons (3 groups for *Acute*RIPC studies and 3 groups for PostC studies), p values by ANOVA (repeated measures) were Bonferroni adjusted. In all cases, $P<0.05$ was considered statistically significant.

7.3 Results

All subjects tolerated the procedures without any complications.

7.3.1 Effect of RIPC on the reperfusion phase of IR injury

IR caused a significant reduction in brachial artery FMD (8.8±1.1% pre- versus 3.6±0.7% post-IR, $p<0.001$; n=10; *figure 7.3a; table 7.1, columns A and B*). 2 RIPC cycles applied on the leg during arm ischaemia protected the endothelium of the brachial artery against IR injury (FMD 9.5±1.2% pre- versus 8.4±1.1% post-IR+*Acute*RIPC*Leg2C*; p=NS; n=8; *figure 7.3b*). Acute protection by RIPC required the completion of a sufficient RIPC stimulus during prolonged ischaemia as 3 RIPC cycles on the arm did not prevent IR-induced reduction in FMD when applied during ischaemia and early reperfusion (FMD 9.3±1.0% pre- versus 3.7±0.7% post-IR+*Acute*RIPC*Arm3C*, $p<0.001$; n=10; *figure 7.3c*). *Acute*RIPC had no effect on blood pressure, heart rate, arterial diameter and FMD flow stimulus during reactive hyperaemia *(table 7.1, columns C to F)*.

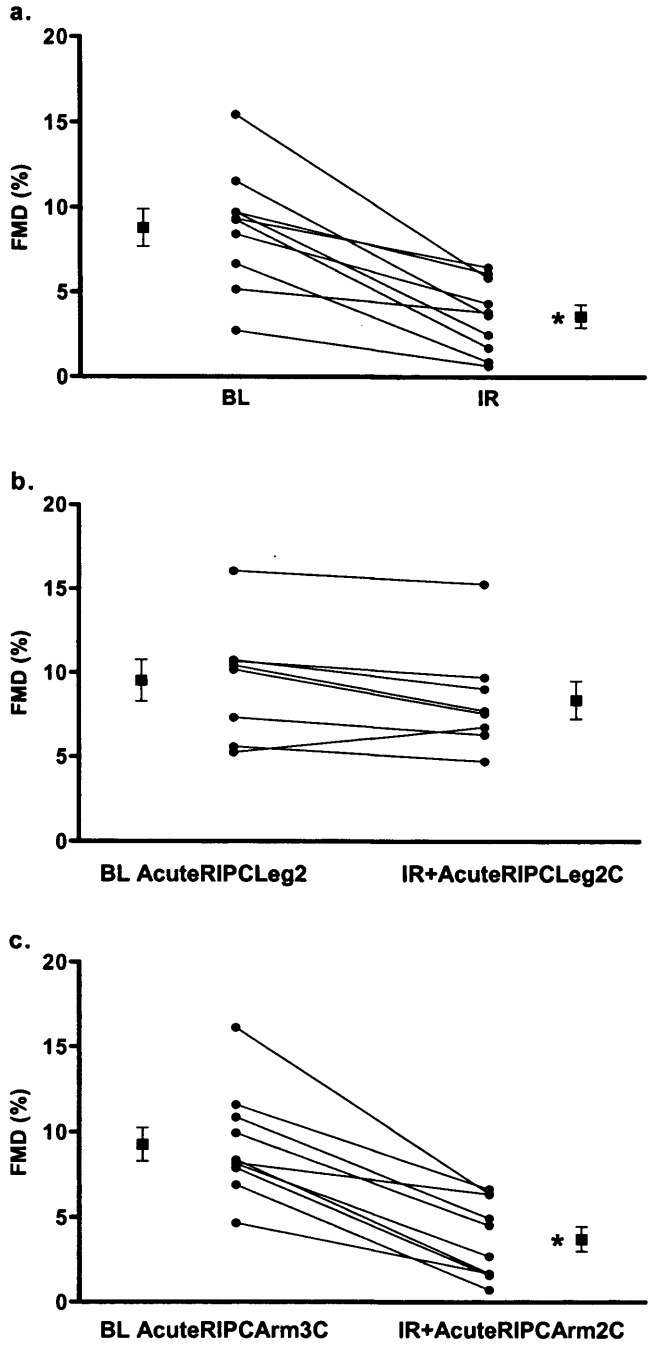


Figure 7.3 Acute protective effect of RIPC against endothelial IR injury. FMD was 8.8±1.1% at baseline (BL) and was reduced by IR (a; IR 3.6±0.7%; *p<0.001, ANOVA; n=10). IR-induced endothelial dysfunction was not observed when 2 RIPC cycles where applied on the contralateral leg during the 20 minute ischaemic insult on the non-dominant arm (*Acute*RIPC*Leg2C*) (b; BL 9.5±1.2% vs. IR+*Acute*RIPC*Leg2C*

8.4±1.1%; p=NS, ANOVA; n=8). In contrast, 3 RIPC cycles applied on the contralateral arm during ischaemia and early reperfusion did not prevent IR-induced endothelial dysfunction (c; BL 9.3±1.0% vs. IR+*Acute*RIPC*Arm3C* 3.7±0.7%; *p<0.001, ANOVA; n=10).

	IR Alone *(n=10)*		***IR+AcuteRIPCLeg2C*** *(n=8)*		***IR+AcuteRIPCArm3C*** *(n=10)*	
	(A) Pre	*(B) Post*	*(C) Pre*	*(D) Post*	*(E) Pre*	*(F) Post*
SBP	122±2	122±2	113±4	111±3	124±3	125±2
DBP	65±2	66±2	62±2	62±2	68±3	68±3
HR	65±2	61±2	64±3	60±3	66±3	64±3
Arterial diameter	3.8±0.2	3.8±0.2	3.4±0.3	3.5±0.3	3.7±0.2	3.8±0.2
Flow stimulus	6.5±1.1	6.8±1.0	7.6±1.6	8.2±1.2	6.6±1.2	7.3±1.1
FMD	**8.8±1.1**	**3.6±0.7 ***	**9.5±1.2**	**8.4±1.1**	**9.3±1.0**	**3.7±0.7 ***

Table 7.1 Summary of pre- and post-IR data from to determine acute protection by RIPC. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter is expressed in millimetres and the FMD flow stimulus is expressed as the peak to baseline volume-flow per minute ratio (no units). Flow mediated dilation (FMD) was expressed in peak % dilation from baseline diameter. * *p<0.001 FMD (A) vs. (B), (B) vs. (D), (E) vs. (F) and (D) vs. (F) (ANOVA).*

7.3.2 Effect of PostC on endothelial IR injury

IR resulted in brachial artery endothelial dysfunction (FMD 9.1±1.2% pre- versus 3.6±0.7% post-IR, p<0.001; n=11; *figure 7.4a; table 7.2, columns A and B*). PostC had no effect on blood pressure, heart rate, arterial diameter and flow stimulus during reactive hyperaemia *(table 7.2, columns C to F)*. PostC, applied immediately at the onset of reperfusion prevented IR-induced endothelial dysfunction (FMD 9.9±1.7% pre- versus 8.3±1.4% post-IR+PostC, p=NS; n=11, *figure 7.4b*). However, no protection was observed when the application of the PostC stimulus was delayed for 1 minute following blood flow restoration to the arm (FMD 10.2±1.7% pre- versus 4.4±1.2% post-IR+*Delayed*PostC, p<0.001; n=7; *figure7.4c*).

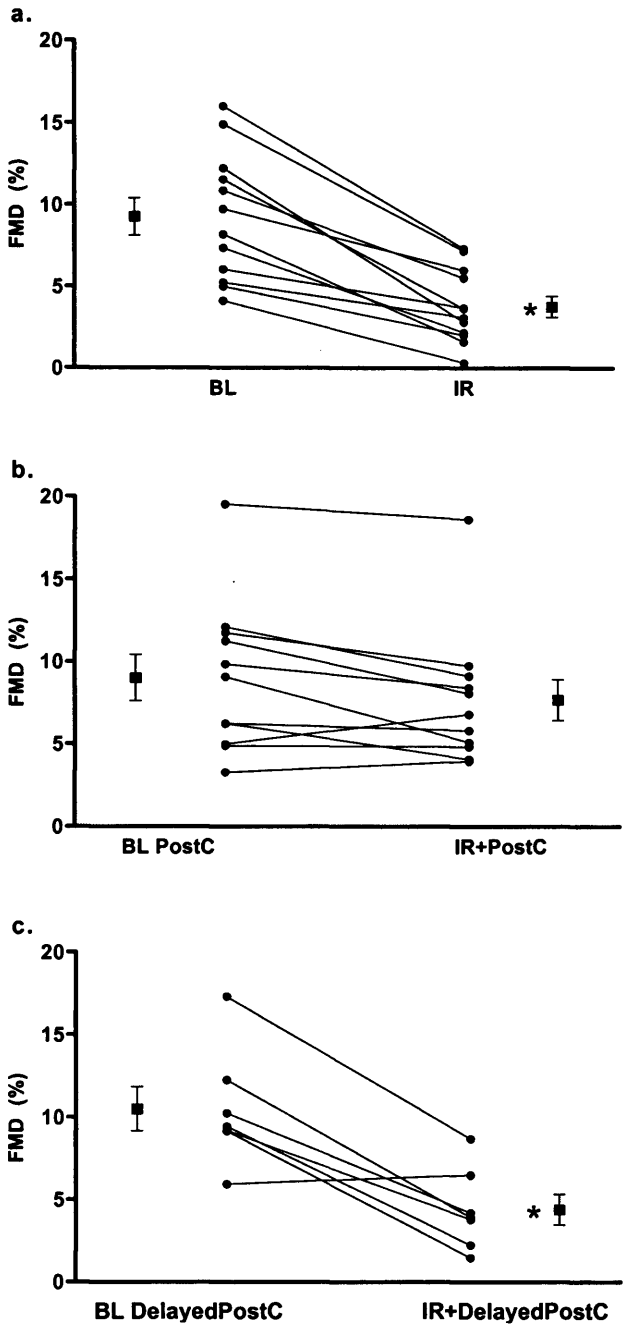


Figure 7.4 Effect of PostC on endothelial IR injury. FMD was 9.1±1.2% at baseline (BL) and was reduced by IR (a; IR 3.6±0.7%; *p<0.001, ANOVA; n=11). Modification of reperfusion by PostC, prevented the reduction in FMD (b; BL 9.9±1.7% vs. IR+PostC 8.3±1.4%; p=NS, ANOVA; n=11). PostC needs to be applied at the onset of reperfusion, as no protection was observed when PostC was induced

after 1 minute of reperfusion (*Delayed*PostC) (c; BL 10.2±1.4% vs. IR+*Delayed*PostC 4.4±1.2%; *p<0.001, ANOVA; n=7).

	IR alone *(n=11)*		***IR+PostC*** *(n=11)*		***IR+DelayedPostC*** *(n=7)*	
	(A) Pre	*(B) Post*	*(C) Pre*	*(D) Post*	*(E) Pre*	*(F) Post*
SBP	112±2	112±2	114±2	115±2	112±3	112±3
DBP	63±1	60±3	65±2	64±2	66±3	64±3
HR	63±2	60±3	67±3	66±3	64±3	61±3
Arterial diameter	3.4±0.2	3.4±0.2	3.2±0.2	3.2±0.2	3.3±0.3	3.4±0.3
Flow stimulus	11.3±1.5	10.9±1.0	8.3±1.7	8.5±1.7	8.0±1.8	8.1±1.4
FMD	**9.1±1.2**	**3.6±0.7** *	**9.9±1.7**	**8.3±1.4†,‡**	**10.2±1.4**	**4.4±1.2** *

Table 7.2 Summary of pre- and post-IR data from studies to determine the effects of PostC on IR injury. SBP/DBP: systolic/diastolic blood pressure (mmHg); HR: heart rate (beats per minute). Arterial diameter was measured in millimetres and the FMD flow stimulus during reactive hyperaemia was expressed as the ratio of peak to baseline volume flow per minute ratio (no units). FMD was expressed as peak % dilation from baseline brachial artery diameter. * *p<0.001 FMD (A) vs. (B) and (E) vs. (F); † p<0.01 FMD (D) vs. (B); ‡ p<0.05 FMD (D) vs. (F) (ANOVA).*

7.4 Discussion

This study shows for the first time in humans, that RIPC, activated during the peri-ischaemic phase of IR, prevents IR-induced endothelial dysfunction. Together with the observation that ischaemic post-conditioning (PostC) causes similar protection, these data suggest that endothelial injury occurs during early reperfusion.

RIPC could be activated co-temporaneously with the ischaemic phase of IR, and provided similar protection to that caused when RIPC was induced in advance of index ischaemia *(see section 3.3.2)*. Endothelial injury as a result of IR is, in theory, a composite of damage during ischaemia and that caused by reperfusion (79). If a significant component of injury occurs during ischaemia, then initiating preconditioning during index ischaemia would be expected to provide less protection against IR injury than when preconditioning was triggered in advance of index ischaemia. However, protection was observed when RIPC was applied during the ischaemic insult and similar to that described in *chapter 3*, when RIPC was initiated in advance of index ischaemia. These data are consistent with the major component of injury occurring at or close to reperfusion (242;407). This was supported by the observation that there was no protection when the preconditioning protocol extended into the reperfusion phase of IR. To specify the relative contribution of the reperfusion phase of IR to ultimate injury with greater precision, I tested the effects of PostC on the endothelial response to IR.

The concept of ischaemic post-conditioning (PostC), which describes protection against IR injury by a series of brief interruptions of reperfusion applied at the very onset of reperfusion, was first introduced in 2003. Studies by Zhao *et al* (546) and

Halkos *et al* (186) demonstrated in a canine model of myocardial infarction that the application a PostC protocol immediately following a prolonged period of sustained coronary artery occlusion, resulted in significant reduction in myocardial infarct size, an effect comparable to that of IPC. These observations were subsequently confirmed by others in a variety of experimental models of IR in the myocardium and other tissues.

The mechanisms of protection by PostC are currently elusive. PostC was initially proposed to be beneficial against IR injury by reducing neutrophil mediated damage during reperfusion (245;546). However, PostC has also been shown to be protective in isolated perfused hearts (150;486;534) and cell culture systems (460) that are neutrophil free, which supports the involvement of additional mechanisms in protection exerted by PostC, including attenuation of reactive oxygen species production (ROS) (186;245;460). Moreover, emerging evidence indicates that protection may be dependent on adenosine receptor stimulation (534), opening of mitochondrial K_{ATP} channels (535), activation of the pro-survival kinases PI3K-Akt (92;486;534) and Erk1/2 (535) and inhibition of mitochondrial permeability transition pore (mPTP) opening (18), key factors implicated in preconditioning-induced protection *(see sections 1.3 and 1.4.3)*. These observations suggest that PostC and IPC have common signalling pathways, which may explain the fact that these protective phenomena are equally effective in protecting against IR injury (186;486).

The results of the present study confirm the endothelial protective effects of PostC in the human vasculature. In the human IR model, the degree of protection by PostC is similar to that achieved by IPC (244) and RIPC *(see section 3.3.2)*, which is in

agreement with animal evidence and confirms that injury suffered during reperfusion is largely responsible for the IR-induced endothelial dysfunction I observed. Most studies indicate that the immediate reperfusion phase is the critical window for the modification of IR injury (245;535). Consistent with this, I observed that PostC was only effective in the first 60 seconds of reperfusion. Taken with the results of *Acute*RIPC, these data suggest that for some ischaemic episodes (in particular those that are not so prolonged as to cause irreversible ischaemic death), the immediate phase of reperfusion is a critical time window to limit reperfusion injury. If these observations in the endothelium can be extrapolated to other organs, they suggest that there is potential for RIPC to reduce tissue injury if initiated during ischaemia and before reperfusion (242).

7.4 Conclusions

The present data demonstrate that RIPC has scope to reduce tissue injury when a sufficient stimulus is administered while an ischaemic event is in progress. Although the mechanism of *Acute*RIPC is not currently clear, the degree of protection was similar to that afforded by PostC applied immediately at the onset of reperfusion, which indicates that *Acute*RIPC may reduce endothelial IR injury by modifying critical events occuring during the first moments of reperfusion.

Chapter 8:

Remote Ischaemic Preconditioning in Paediatric Renal Transplantation

8.1 Introduction

Kidney transplantation has emerged as a treatment option for patients with end-stage renal disease (ESRD) and offers the greatest potential for restoring a healthy, productive life in these individuals (165). The main focus of transplantation therapy has been the prevention of acute rejection (AR); the introduction of more stringent cross-matching criteria and development of novel immunosuppressive regimes have lowered early rates of AR to below 20% and increased one year renal allograft survival to over 80% (293;444). Despite improvements in donor and recipient management, as well as in diagnostic and therapeutic modalities, up to 50% of renal allografts from cadaveric donors (4;363) and approximately 5% from live-related donors (4;434) manifest a degree of early functional impairment, which in referred to clinically as delayed graft function (DGF). DGF is a form of acute renal failure and its clinical manifestations vary along a spectrum of severity, from subtle slowing of the expected decline in plasma creatinine, to prolonged oliguria/anuria requiring dialysis following transplantation (363;385).

Ischaemia and reperfusion (IR) injury suffered by the kidney during the transplantation process has a key role in the development of DGF (334). Overall ischaemia of a renal graft is the sum of a possible transient warm ischaemic interval before or during actual removal from the donor, cold ischaemia associated with preservation and storage, and that occuring during the period of revascularisation to the recipient's circulation (475). The duration of ischaemia has been shown to be an independent risk factor for DGF, with a 23% increase in risk for every six hours of ischaemia (363). Prolonged ischaemia and subsequent reperfusion result in injury to the kidney, via mechanisms that are similar to those described for the myocardium

(see section 1.2.2) (277;475), and can lead to acute tubular necrosis (ATN), with subsequent graft dysfunction (385). The outer medulla of the kidney is mainly affected by IR (66), with the distal portion (S3 segment) of the proximal tubule being the primary site of injury (304;499), due to its marginal oxygenation under normal physiologic conditions coupled with increased metabolic demand (8;122;304). Moreover, IR can increase graft immunogenicity by enhanced expression of major histocompatibility complex (MHC) class I and II antigens (437;445), which can elicit a strong T-cell mediated immune response and increase the risk of acute rejection (AR). AR has been shown to be more frequent in transplant patients that experience DGF compared to those with immediate graft function (211;358), which is consistent with IR-mediated increase in acute recipient immunological reactivity (314).

Ultimately, IR leads, either directly or through "priming" of the immune system, to a reduction of functioning renal mass, and emerging evidence suggests that the degree of IR injury during transplantation may be a determinant of long-term graft survival (187;475). In this respect, DGF is associated with a reduction in the half-life of renal grafts from 11.5 to 7.2 years (188) and analysis from the US Renal Data System, involving more than 37,000 renal transplant patients, revealed that DGF was associated with increased risk of five-year graft loss (relative risk 1.53) (363). Thus, protective strategies aiming to attenuate IR injury during transplantation may be beneficial not only for early graft function, but could also improve long-term graft survival (385). In a double-blind, placebo-controlled clinical trial in renal transplantation, reduction of reactive oxygen species (ROS)-mediated injury during IR, by administration of the anti-oxidant enzyme superoxide dysmutase (SOD) prior

to reperfusion, resulted in significant reduction in the incidence of acute and chronic rejection and improved outcome (276).

Another strategy that could prove effective in limiting the deleterious effects of IR injury to the kidney in the clinical setting of renal transplantation is ischaemic preconditioning (IPC) (59). In animal models, local and remote IPC (RIPC) reduce injury and preserve renal function following IR injury to the kidney (20;410). IPC has also been shown to attenuate IR injury in isolated human proximal tubular cells (287). Although the signal transduction pathway of IPC has been extensively studied in the myocardium *(see section 1.3)*, there is currently limited data on the mechanisms of renal IPC (59). Similar mediators (adenosine) (285), second messengers (protein kinase C, mitogen-activated protein kinase) (286;287) and end effectors (heat shock protein 27, 70; inducible nitric oxide synthase) (22;222) to those implicated in non-renal tissues have been described. Only one study to date has investigated the potential protective effects of IPC in renal transplantation, and has demonstrated a clear protective effect in the rat (484). However, there are no data on the potential protective effects of RIPC in the setting of renal transplantation.

Despite the emerging body of evidence demonstrating the effect of IR injury on early function and survival of kidney grafts after transplantation, reliable parameters for the assessment of renal ischaemic damage have not yet been defined. DGF, generally accepted as the clinical expression of IR injury in the kidney, can also be caused by post-transplant infection or the use of certain nephrotoxic immunosuppressive compounds, which limit the specificity of DGF as a measure of IR injury alone (445). In addition, DGF is a relatively non-sensitive marker of IR-induced injury to the renal

graft, due to the wide spectrum of clinical manifestations it includes (385). Thus, in order to design a study to investigate the potential protective effects of a strategy, such as RIPC, against kidney IR injury in renal transplantation, it would be essential to assess the value of surrogate markers of renal ischaemic injury.

Given the sensitivity of the proximal tubule to IR, evaluation of proximal tubular function following transplantation may provide a reliable measure of renal IR injury. Retinol binding protein (RBP) is a low molecular weight protein that is freely filtered by the glomerulus and is normally 99.9% reabsorbed by the proximal tubule (481). As a result, it is only found in very small quantities in urine from healthy individuals and diseases that affect the proximal tubule (e.g. Fanconi syndrome) are associated with increased excretion of RBP in the urine *(see section 2.2.1.1)*. Consequently, it would be expected that IR-induced proximal tubule injury would lead to a rise in the urinary concentration of RBP post-transplantation (335;403;481). In order to test this hypothesis, I performed a pilot study in paediatric patients undergoing cadaveric and live-related renal transplantation, the results of which are presented in the first part of this chapter. RBP data obtained from the pilot study were subsequently used to design a randomised, double-blind clinical trial aiming to determine the potential protective effects of second window RIPC against IR injury in paediatric live-related renal transplantation, using urinary RBP concentration as a marker of transplantation-induced IR injury. For the latter study the hypothesis was that RIPC, administered 24 hours prior to transplantation, would result in protection against transplantation-induced IR injury to the renal graft. The latter study is currently in progress, and in the second part of this chapter I will present preliminary data from the first 10 paediatric patients recruited in the trial.

8.2 Methods

8.2.1 Patients

23 consecutive paediatric renal transplant patients (15 male, 7 female; mean age±SD 12.1±4.6 years, range 3 to 17) were recruited from renal transplant clinics at Great Ormond Street Hospital for Children NHS trust. 8 patients received a renal graft from cadaveric donors and 15 from live-related donors. Primary diagnoses for patients included in the study are summarised in *table 8.1*. Studies were approved by the Institute of Child Health and Great Ormond Street Hospital research ethics committee and informed consent was obtained from all study participants (or from participants' parents when patients were less than 16 years old).

Primary diagnosis *(n)*	
Congenital and hereditary disease *(13)*	***Acquired disease*** *(10)*
Autosomal recessive polycystic renal disease *(1)*	ESRF 2^0 to birth asphyxia *(1)*
Congenital nephrotic syndrome *(1)*	ESRF 2^0 to CyA toxicity *(2)*
Nephronopthisis (medullary cystic disease) *(1)*	ESRF 2^0 to interstitial nephritis *(1)*
Posterior ureteral valves *(1)*	ESRF 2^0 to microscopic polyangitis *(1)*
Renal dysplasia *(7)*	Focal segmental glomerulosclerosis *(3)*
Vesico-ureteric reflux *(2)*	Hypertension *(1)*
	Renal venous thrombosis *(1)*

Table 8.1 Summary of primary diagnoses for study participants. *ESRF: end-stage renal failure; CyA: cyclosporin A.*

8.2.2 Transplantation

Renal grafts were retrieved by open surgery in cadaveric donors and laparoscopically in live-related donors. Grafts were cold stored in hypertonic citrate preservation solution (Marshall/HOC; n=22) or University of Wisconsin preservation solution (n=1) until transplantation. Transplantation procedures in all patients were performed by two surgeons (Mr. G. Koffman, Guys Hospital NHS trust, and Miss R. Lord, Royal Free Hospital NHS trust). Post-operative management was performed according to a standardised protocol. The post-transplantation immunosuppressive regime included azathioprine (n=20), mycophenolate mofetil (n=2), tacrolimus (FK506; n=20), cyclosporin (n=2), and methyl prednisolone (n=21).

8.2.3 Pilot study: Urinary RBP as a marker of IR injury in renal transplantation

13 patients undergoing renal transplantation [8 cadaveric (CAD), 5 live-related (LRD)] took part in a pilot study to characterise urinary RBP as a marker of IR injury to the kidney. Blood and urine samples were collected and processed as described in *sections 2.2.1.3.1* and *2.2.2.2.1*. The first set of samples was obtained immediately following transplantation, while patients were recovering from general anaesthesia (t=0 hours). Subsequent samples were collected 8-hourly for the first 3 days post-transplantation (t=72 hours) and then once daily until the eighth post-operative day (t=192 hours) *(figure 8.1)*.

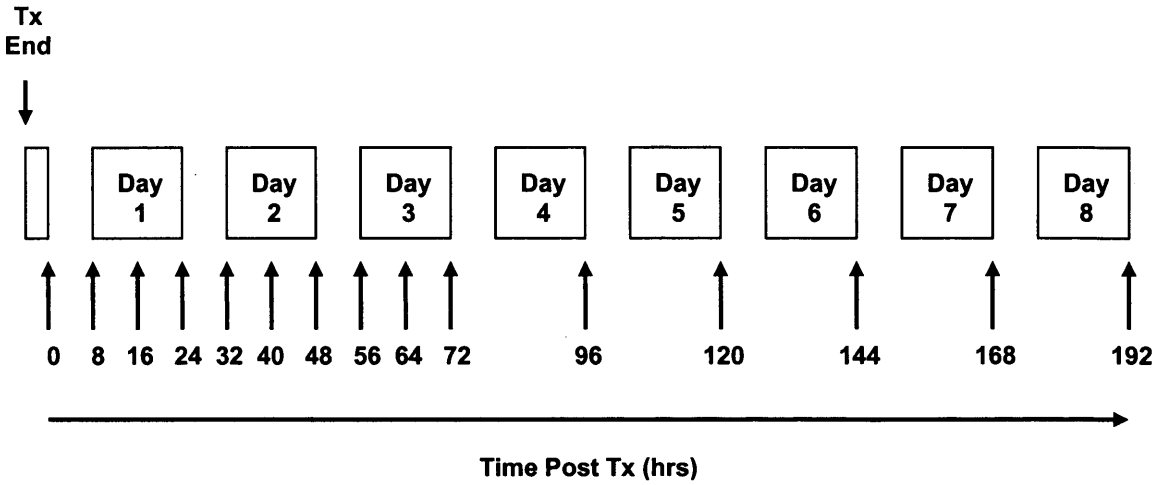


Figure 8.1 Pilot study blood and urine sample collection schedule. Samples were collected from recipients immediately following the end of transplantation (Tx), then 8-hourly for the first 72 hours (days 1 to 3) and at 96, 120, 144, 168 and 192 hours post-transplantation (days 4 to 8). 4 ml of blood and 5 ml of urine were collected at each time point (15 time points in total).

8.2.4 Effect of RIPC against IR injury during live-related renal transplantation

10 renal transplant patients and respective live-related donors took part in a preliminary study to determine the potential protective effects of RIPC against IR injury suffered by the kidney during transplantation. Donor-recipient pairs were randomised to RIPC and control groups by sealed envelopes.

RIPC was induced by applying 3 cycles of 5-minutes ischaemia and reperfusion on the arm, as described in *section 3.2.3*. To maximise the chances of observing an effect, RIPC was administered to both the live-related donor and recipient. Application of the RIPC stimulus was performed 24 hours prior to renal graft harvest and transplantation *(figure 8.2)*. In donor-recipient pairs randomised in the control

group, a blood pressure cuff was placed in the upper part of the arm, but was inflated to a pressure of 40mmHg, and thus did not result in arm ischaemia (sham RIPC). Three 5-minute inflations and deflations were applied on both donor and recipient 24 hours prior to kidney harvest and transplantation respectively, as for the RIPC group.

Baseline blood and urine samples were obtained from donors and recipients prior to administration of RIPC or sham RIPC. Subsequent samples were collected from recipients only, immediately following transplantation as described in *section 8.2.3.* Results from live-related transplant patients included in the pilot study demonstrated that urinary RBP reached its lowest concentration within 72 hours following transplantation *(see section 8.3.1.2).* Consequently, blood and urine samples in the RIPC study were collected for the first 72 hours post-transplantation (days 1 to 3) *(figure 8.2).*

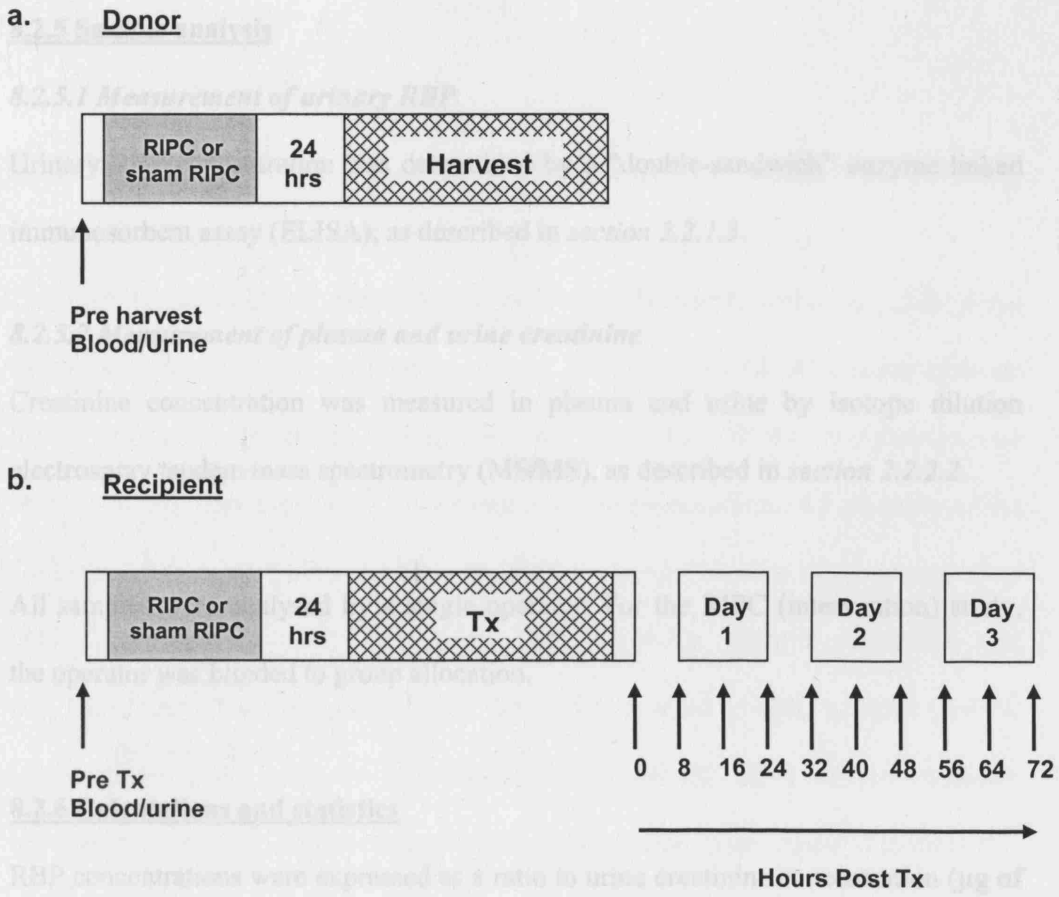


Figure 8.2 Experimental protocol of studies to determine the effects of RIPC against IR injury during live-related renal transplantation. RIPC [3-cycles of 5-minutes arm ischaemia (blood pressure cuff inflation to 200mmHg) and reperfusion], or sham RIPC (control; same as RIPC but blood pressure cuff inflated to 200mmHg) were applied on both the donor (a) and the recipient (b), 24 hours before harvest and transplantation (Tx) respectively. Blood and urine samples were collected prior to RIPC or sham RIPC administration from both donor and recipient (a, b). Subsequent samples were collected from recipients only, at 8-hour intervals, for the first 72 hours post transplantation (b).

8.2.5 Sample analysis

8.2.5.1 Measurement of urinary RBP

Urinary RBP concentration was determined by a "double-sandwich" enzyme-linked immunosorbent assay (ELISA), as described in *section 2.2.1.3.*

8.2.5.2 Measurement of plasma and urine creatinine

Creatinine concentration was measured in plasma and urine by isotope dilution electrospray tandem mass spectrometry (MS/MS), as described in *section 2.2.2.2.*

All samples were analysed by a single operator. For the RIPC (intervention) study, the operator was blinded to group allocation.

8.2.6 Calculations and statistics

RBP concentrations were expressed as a ratio to urine creatinine concentration (μg of RBP per mmol of creatinine) *(section 2.2.1.3.4).* Plasma creatinine concentration was expressed in μmol/l. Curves of urinary RBP concentration against time were constructed and the area under the curve (AUC) for the first 72 hours post-transplantation was calculated. Mean AUC and standard deviation values for live-related patients in the pilot study *(section 8.3.1.2)* were used to perform a power calculation for the RIPC (intervention) study *(see section 8.3.1.5).* Data was compared using student's *t*-test (unpaired) or one-way analysis of variance (ANOVA). RBP against time and plasma creatinine against time curves for pilot or intervention study groups were compared by 2-way ANOVA. For multiple comparisons, p values by ANOVA were Bonferroni adjusted. Correlations between continuous variables were assessed by the Pearson correlation test. In all cases, $p<0.05$ was considered statistically significant.

8.3 Results

8.3.1 Pilot study: Urinary RBP as a marker of IR injury in renal transplantation

8.3.1.1 Patient group characteristics

Demographic and clinical details for cadaveric (CAD) and live-related (LRD) renal transplant patients are summarised in *table 8.2*. There were no differences between the two groups in age, height, weight and body mass index (BMI). Two patients, one in each group, had previously undergone renal transplantation. All patients in the CAD group were on peritoneal dialysis (PD) pre-transplantation (Tx), compared to only 2 patients in the LRD group. Mean pre-Tx plasma creatinine was 619.3±51.9μmol/l in CAD patients and 503.8±117.3μmol/l in LRD patients (p=0.30 versus CAD). There were no differences in donor age and HLA antigen mismatch between the groups. Cadaveric donor clinical information is summarised in *table 8.3*. Renal grafts from cadaveric donors were subjected to significantly longer periods of ischaemia compared to grafts from live-related donors (CAD 20.9±2.2 hours versus LRD 3.2±0.6 hours, $p<0.0001$).

	CAD (n=8)	LRD (n=5)	p
Sex (m/f)	5/3	4/1	—
Age (yrs)	11.5±2.1	12.4±1.5	0.77
Height (cm)	130.1±9.9	133.8±5.5	0.79
Weight (kg)	38.9±6.7	38.9±4.0	0.99
Body Mass Index (kg/m^2)	20.8±2.0	21.2±1.8	0.89
Type of renal disease (congenital/acquired)	4/4	3/2	—
Previous renal Tx (n/total)	1/8	1/5	—
Pre Tx dialysis (on dialysis/total)	8/8 (PD)	2/5 (PD)	—
Plasma creatinine pre Tx (μmol/l)	619.3±51.9	503.8±117.3	0.30
Donor age (yrs)	37.8±3.0	40.8±0.9	0.46
HLA mismatch	1.6±0.3	2.0±0.6	0.57
Ischaemia time (hrs)	**20.9±2.2**	**3.2±0.6**	<0.0001

Table 8.2 Summary of demographic and clinical details for cadaveric (CAD) and live-related (LRD) renal transplant (Tx) patients included in the pilot study. Continuous variables are expressed as mean±SE and were compared by unpaired student's *t*-test.

	Sex	Age (yrs)	Clinical details	Plasma creatinine at harvest (μmol/l)
1	F	20	Fatal head injury (RTA), prolonged hypotension pre harvest	124
2	F	45	Subarachnoid haemorrhage, 1 episode of hypotension (90/45) and 1episode of hypertension (180/100) pre harvest	54
3	M	34	Subarachnoid haemorrhage, out of hospital cardiac arrest	95
4	M	37	Pneumococcal meningitis (*)	102
5	M	48	Subarachnoid haemorrhage, 1 episode of hypotension (60/40) pre harvest	72
6	F	43	Subarachnoid haemorrhage, 1 episode of hypertension (210/110) pre harvest	76
7	F	38	Fatal head injury (RTA)	55

Table 8.3 Summary of cadaveric donor details. All donors were heart-beating donors. The cause of death and adverse cardiovascular events during the pre-harvest period are reported for each patient. *(*) renal graft donor to two CAD patients included in the study; RTA: road traffic accident.*

8.3.1.2 Urinary RBP following renal transplantation

In the CAD group, urinary RBP concentration immediately following Tx (t=0) was 48,033±10,464μg/mmol of creatinine (Cr) and was significantly reduced thereafter ($p<0.0001$, ANOVA; *figure 8.3a*). The lowest RBP concentration was observed 72 hours post-Tx (4,058±2,007μg/mmol of Cr, $p<0.001$ versus t=0). In LRD patients, urinary RBP concentration was 14,353±1,303μg/mmol of Cr immediately post-operatively. RBP levels were subsequently reduced ($p<0.0001$, ANOVA; *figure 8.3b*), and the lowest concentration was observed at 72 hours post-Tx (326±34μg/mmol of Cr, $p<0.001$ versus t=0), remaining relatively constant thereafter.

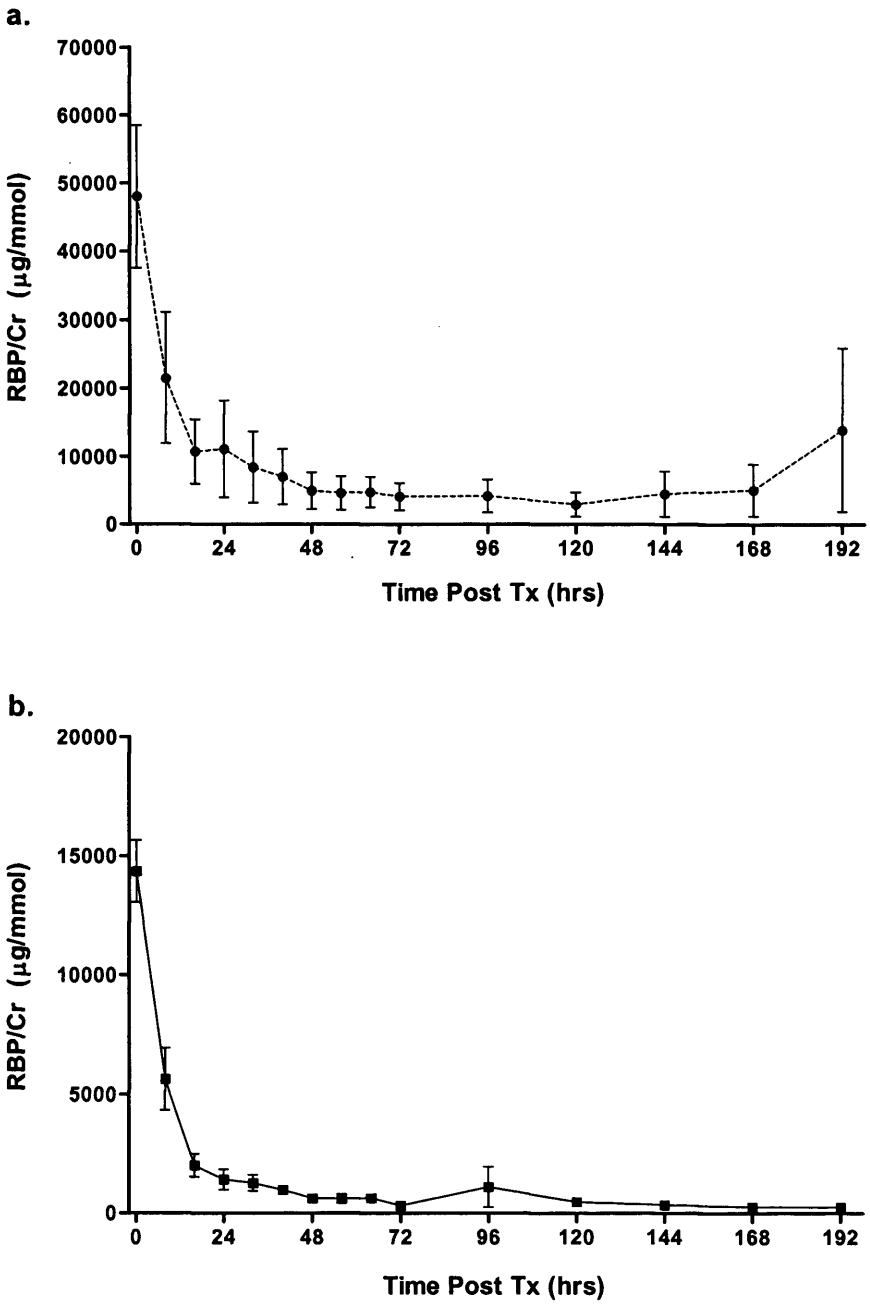


Figure 8.3 Urinary RBP concentration following cadaveric (a) or live-related renal transplantation (b). RBP concentration was expressed as a ratio to urinary creatinine concentration (RBP/Cr; µg/mmol of Cr).

The urinary RBP against time post-Tx curve for cadaveric transplant patients was significantly different to the one for live-related patients ($p<0.0001$, ANOVA; *figure 8.4a*). RBP concentration immediately post-Tx ($t=0$) was significantly higher in the CAD group ($p<0.001$ versus LRD, ANOVA), but no significant differences were found at any other time points. In addition, there was a significant difference in the area under the curve (RBP against time) for the first 72 hours post-Tx (AUC RBP/Cr 72hrs) between CAD and LRD groups (CAD 524,769±110,341 versus LRD 164,669±19,459, $p<0.05$; *figure 8.4b*). There was a good correlation between AUC RBP/Cr 72 hrs and ischaemia time ($r=0.72$, $p<0.01$; *figure 8.4c*).

8.3.1.3 Plasma creatinine following renal transplantation

Post-Tx plasma creatinine profiles for cadaveric and live-related renal transplant patients are shown in *figure 8.5a*, demonstrating a significant reduction in creatinine concentration with time ($p<0.0001$, ANOVA). The creatinine against time curve for cadaveric transplant patients was different to the one for live-related patients ($p<0.0001$, ANOVA), but comparison of creatinine concentrations between the two groups at individual time points did not demonstrate any significant differences. The time for plasma creatinine to halve for CAD and LRD groups was 129.4±44.9 hours and 10.8±2.1 hours respectively *(figure 8.5b)*, but the difference between the two did not reach statistical significance ($p=0.06$). Time for plasma creatinine to halve correlated well with ischaemia time ($r=0.71$, $p<0.01$; *figure 8.5c*).

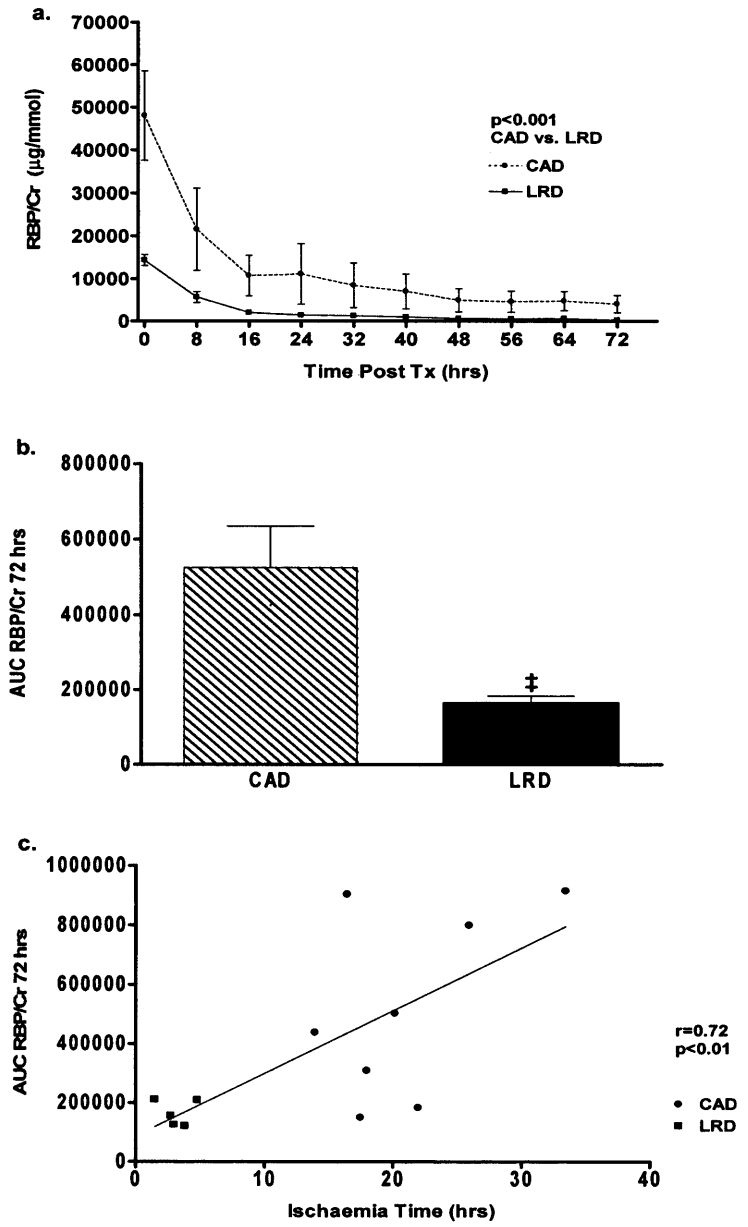


Figure 8.4 (a) Graph showing urinary RBP concentration (expressed as a ratio to urinary creatinine concentration; RBP/Cr) in cadaveric (CAD) and live-related (LRD) renal transplant patients for the first 72 hours post-transplantation (Tx) ($p<0.0001$ CAD vs. LRD, 2-way ANOVA). (b). Area under the time curve for RBP for the first 72 hours following Tx (AUC RBP/Cr 72 hrs; † $p<0.05$ CAD vs. LRD, unpaired *t*-test). (c). Graph of AUC RBP/Cr 72 hrs against ischaemia time (Pearson $r=0.72$, $p<0.01$).

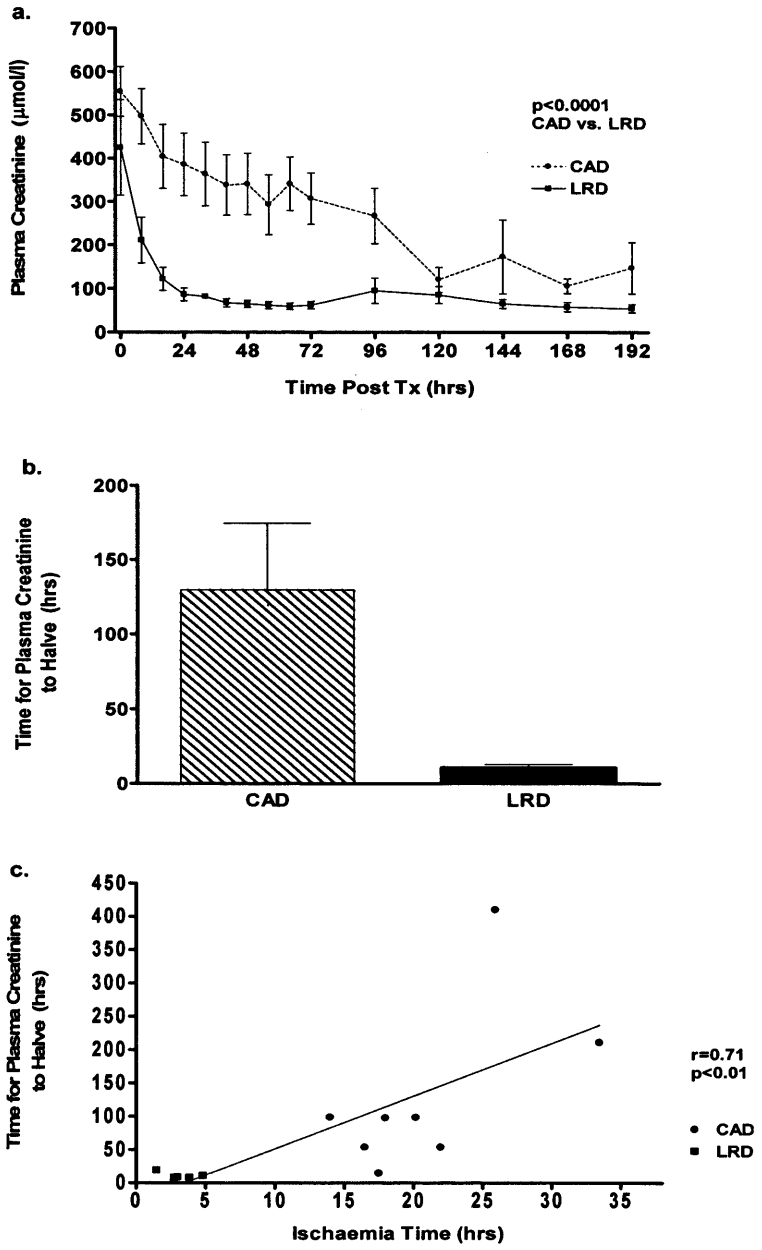


Figure 8.5 (a) Plasma creatinine concentration profile following cadaveric (CAD) or live-related (LRD) renal transplantation (Tx) ($p<0.0001$ CAD vs. LRD, 2-way ANOVA). (b). Time for plasma creatinine to halve (in hours) for CAD and LRD groups ($p=0.06$, unpaired *t*-test). (c). Graph of time for plasma creatinine to halve against ischaemia time (Pearson $r=0.71$, $p<0.01$).

8.3.1.4 Transplantation outcome

Three cadaveric renal transplant patients presented with delayed graft function (DGF) post-operatively, as assessed by the presence of anuria or oliguria and the need for dialysis following transplantation. In contrast, urine production was immediate in all live-related renal transplant patients. Episodes of acute rejection were reported in three CAD patients, but did not occur in LRD patients. Plasma creatinine one month post-Tx was 82.6±9.6μmol/l in the CAD group, and was similar to plasma creatinine concentration in the LRD group (82.6±6.8μmol/l, p=0.99 versus CAD). There was no correlation between the area under the curve for urinary RBP against time for the first 72 hours post-Tx (AUC RBP/Cr 72 hrs) and plasma creatinine levels 1 month following transplantation (r=0.37, p=0.21).

8.3.1.5 Power calculation for RIPC study

The mean and standard deviation for AUC RBP/Cr 72 hrs in the live-related transplant group (*section 8.3.1.2)* was used to perform a power calculation in order to determine the appropriate number of LRD patients to be recruited in the RIPC (intervention) study. Given a mean value for AUC RBP/Cr 72 hrs of 164,669 (standard deviation 43,511), I calculated that 20 LRD patients (10 in each group) would be required to demonstrate that RIPC can reduce post-Tx urinary RBP excretion (expressed as AUC RBP/Cr 72 hrs) by 30%. This was based on an α value of 0.05 and β level of 0.9. Data presented in *section 8.3.2* are preliminary data from the first ten patients recruited in the study.

8.3.2 Effect of RIPC against IR injury during live-related renal transplantation

8.3.2.1 Patient group characteristics

Demographic and clinical details for live-related renal transplant patients in the control and RIPC groups are summarised in *table 8.4*. There were no differences between the two groups in age, height, weight and body mass index (BMI). One patient in the RIPC group had previously undergone renal transplantation. 3 patients in the control group were on peritoneal dialysis (PD) pre-Tx, compared to 2 patients in the RIPC group. Mean pre-Tx plasma creatinine was 725.8±161.6μmol/l in control patients and 560.8±157.9μmol/l in RIPC patients (p=0.49 versus control). Mean urinary RBP concentration was 7,409.2±882.7 and 8,435.5±3,813.0μg/mmol of Cr for control and RIPC recipients respectively (p=0.83 control versus RIPC). There were no differences in donor age and HLA antigen mismatch between the groups. Mean urinary RBP was 12.9±2.2 and 7.0±2.2μg/mmol of Cr for donors in control and RIPC groups respectively (p=0.11 control versus RIPC). Renal grafts in control and RIPC groups were subjected to similar periods of ischaemia (control 2.0±0.8 hours versus RIPC 3.2±0.9 hours, p=0.34).

	Control (n=5)	RIPC (n=5)
Sex (m/f)	3/2	3/2
Age (yrs)	11.0±1.9	13.8±1.8
Height (cm)	155.7±6.4	141.6±11.2
Weight (kg)	44.2±6.8	73.2±25.9
Body Mass Index (kg/m^2)	22.1±0.8	20.5±4.0
Type of renal disease (congenital/acquired)	3/2	2/3
Previous renal Tx (n/total)	0/5	1/5
Pre Tx dialysis (on dialysis/total)	3/5 (PD)	2/5 (PD)
Urinary RBP pre Tx (µg/mmol of Cr)	7,409.2±882.7 (n=3)	8,435.5±3813.0 (n=4)
Plasma creatinine pre Tx (µmol/l)	725.8±161.6	560±157.9
Donor age (yrs)	41.0±3.6	44.8±1.8
Donor urinary RBP pre Tx (µg/mmol of Cr)	12.9±2.2	7.0±2.2
HLA mismatch	2.8±0.2	2.0±0.3
Ischaemia time (hrs)	2.0±0.8	3.2±0.9

Table 8.4 Summary of demographic and clinical details for LRD patients in control and RIPC groups.

8.3.2.2 Effect of RIPC on urinary RBP following transplantation

The urinary RBP against time post-Tx curve for control LRD patients was significantly different to the one for patients in the RIPC group ($p<0.01$, ANOVA; *figure 8.6a*). RBP concentration immediately post-Tx (t=0) was significantly higher in the control group (control 14,170±3,727μg/mmol of Cr versus RIPC 7,598±2132μg/mmol of Cr, $p<0.01$, ANOVA), but no significant differences were found at any other time points. The mean area under the curve (RBP against time; AUC RBP 72 hrs) was 260,482±61,233 in control patients and was reduced by approximately 40% in the RIPC group (146,334±52,492), but the difference did not reach statistical significance ($p=0.19$; *figure 8.6b*).

8.3.2.3 Effect of RIPC on plasma creatinine following transplantation

The plasma creatinine against time curve for control patients was different to the one for RIPC patients ($p<0.001$, ANOVA; *figure 8.7a*), but comparison of creatinine concentrations between the two groups at individual time points demonstrated a significant difference only immediately post-Tx (t=0) (control 637.8±145.1μmol/l versus 298.2±67.3μmol/l, $p<0.01$, ANOVA). The time for plasma creatinine to halve for control and RIPC groups was 10.3±1.4 hours and 6.4±1.5 hours respectively *(figure 8.7b)*, but the difference between the two was not statistically significant ($p=0.09$).

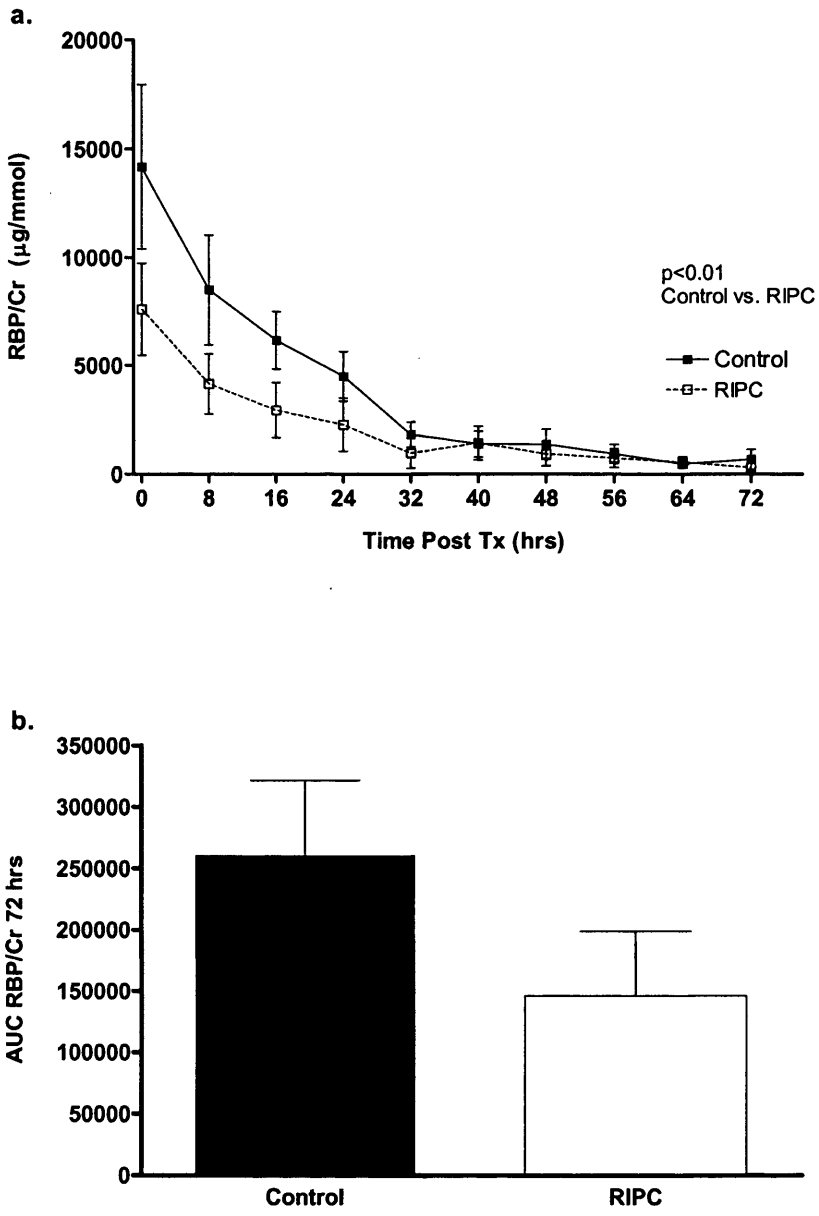


Figure 8.6 Effect of RIPC on urinary RBP following live-related transplantation (Tx). (a) Graph showing urinary RBP concentration (expressed as a ratio to urinary creatinine concentration; RBP/Cr) in control and RIPC patients ($p<0.01$ control vs. RIPC, 2-way ANOVA). (b). Area under the time curve for RBP in control and RIPC groups (AUC RBP/Cr 72 hrs; $p=0.19$ control vs. RIPC, unpaired *t*-test).

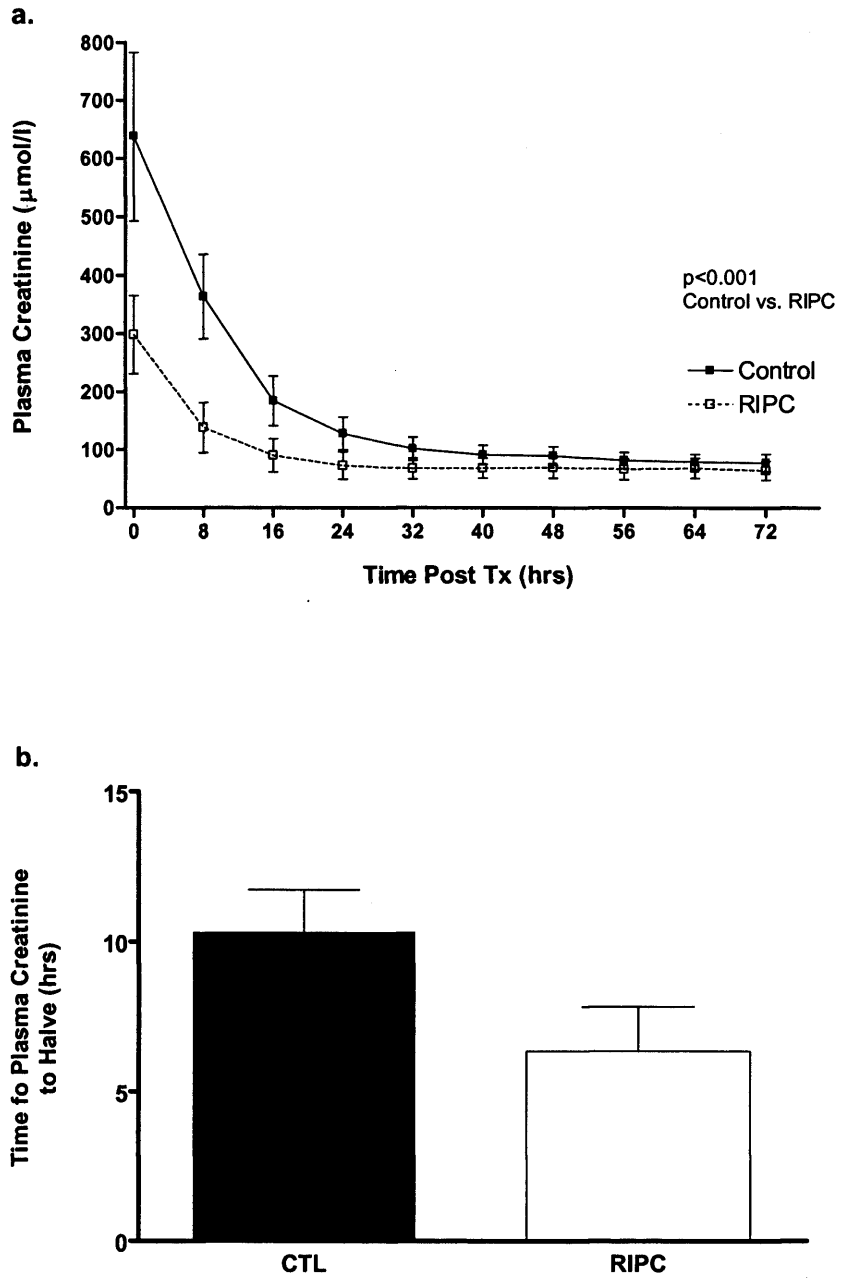


Figure 8.7 Effect of RIPC on plasma creatinine concentration following live-related renal transplantation (Tx). (a) Plasma creatinine profiles for control and RIPC groups ($p<0.001$ control vs. RIPC, 2-way ANOVA). (b) Time for plasma creatinine to halve (hours) in control and RIPC renal Tx patients ($p=0.09$ control vs. RIPC, unpaired *t*-test).

8.3.2.4 Transplantation outcome

One patient in the control group was anuric immediately following transplantation, which was consistent with delayed graft function. Urine production was immediate in all other patients and no episodes of acute rejection were reported. Plasma creatinine 1 month post-Tx was 121.6±27.9μmol/l in the control group and 75.8±27.9μmol/l in the RIPC group (p=0.21 RIPC versus control).

8.4 Discussion:

The present study provides preliminary evidence that RIPC of the limb provides late protection against IR injury in paediatric patients undergoing paediatric renal transplantation. The data presented in this chapter are the results of an interim analysis performed following recruitment of the first ten patients. The study is currently in progress and recruitment of the 20 patients required *(see section 8.3.1.5)* is expected to be completed by late October 2005.

IR injury following renal transplantation was assessed by measuring the concentration of retinol binding protein (RBP) in the urine of renal allograft recipients. RBP was chosen as a marker based on the results of a pilot study that included patients receiving a kidney from a cadaveric or live-related donor. In addition to RBP, several other candidate compounds were assessed in the urine of these patients, including trimethylamine-N-oxide (TMAO), dimethylamine (DMA), lactate and citrate, all of which have been proposed as markers of IR injury in animal models of renal transplantation (193). Data for these putative markers are not presented in this chapter as no significant changes in the urinary concentrations were observed following human renal transplantation. Moreover, no differences in the levels of these compounds were observed between cadaveric and live-related transplant patients. Any putative marker of renal IR injury should be able to differentiate between allografts from cadaveric or live-related donors, as the former are subjected to considerably longer periods of ischaemia following harvest and thus suffer a greater degree of ischemic injury (90;474).

In contrast to other candidate markers, urinary RBP levels were significantly increased following transplantation in children, indicating tubular dysfunction in the renal graft. This is in agreement with what has been previously reported in adults (335;403). In live-related transplant patients, the concentration of RBP in the urine immediately after the operation was approximately one thousand times greater than the value reported in healthy children *(see section 2.2.1.1)* (450). In cadaveric transplant patients, urinary RBP concentration was five thousand times greater than normal (450) and was significantly higher than in live-related transplant patients. In both patient groups, the excretion of RBP in the urine was decreased for the first 72 hours post-operatively, indicating recovery of tubular function, with levels remaining relatively constant thereafter (but still higher than normal). The area under the time curve for RBP, for the first 72 hours following transplantation, was greater in cadaveric compared to live-related transplant patients, an observation that could only be attributed to the significantly longer ischaemia time in grafts from cadaveric donors, as all other relevant clinical parameters were similar between the two groups *(see section 8.3.1.1)*. In addition, the area under the curve for RBP correlated well with ischaemia time, which is consistent with urinary RBP being a marker of renal IR injury in this setting. Interestingly, ischaemia time also correlated with graft glomerular function following transplantation, as assessed by the time required for plasma creatinine to halve. Recovery of glomerular function in grafts from cadaveric donors was slower compared to grafts from live-related donors, although the difference did not reach statistical significance *(see section 8.3.1.3)*.

In the intervention part of the study, the effect of RIPC against clinical IR injury was tested in patients undergoing live-related renal transplantation. This patient group was

chosen because procedures are planned in advance and this facilitates participant recruitment. In addition the ischaemic insult to the renal graft is relatively constant, allowing straightforward comparisons to be made between control and RIPC groups. Interim analysis of data obtained from the first ten patients recruited in the study demonstrated that RIPC resulted in approximately 40% reduction in RBP excretion in the urine for the first 72 hours following transplantation, as assessed by the area under the time curve for urinary RBP. The observed reduction in urinary RBP concentration in preconditioned patients could not be attributed to pre-transplantation differences in renal graft tubular function, as similar levels of RBP were observed in the urine of donors in both study groups 24 hours before harvest. Moreover, no differences were found in any other relevant clinical parameters (including ischaemia time) between control and RIPC groups *(see section 8.3.2.1)*. Thus, the present results are consistent with a beneficial effect of RIPC against IR injury suffered by the renal graft during transplantation.

In addition to the beneficial effects of RIPC against renal tubular injury, creatinine data presented in this chapter suggest that RIPC may also improve glomerular function in the graft following transplantation. Time for plasma creatinine to halve in the RIPC group was reduced by approximately 40% compared to control, but the difference did not reach statistical significance. However, baseline plasma creatinine in the control group was higher compared to the one for the RIPC group, and it is possible that the observed results for time for plasma creatinine to halve could be attributed to this baseline difference. However, no correlation was found between the two parameters ($r=0.05$, $p=0.88$), which further suggests that the improved recovery of glomerular function in the RIPC groups is due to a direct effect of RIPC to limit IR

injury to the renal graft. Moreover, RIPC could be beneficial for graft function in the long term, as plasma creatinine in RIPC patients one month following transplantation was lower compared to control patients, although the latter remains to be confirmed.

In the current study, RIPC was administered on both donor and recipient 24 hours prior to harvest and transplantation respectively. Second window RIPC was feasible due to the planned nature of the procedure. This approach was chosen as the application of the RIPC stimulus could be performed in the ward well in advance of transplantation, and would therefore not interfere with anaesthetic and surgical protocols, as would be the case if RIPC was administered immediately post-operatively (in the anaesthetic room). RIPC was induced in both donor and recipient in order to maximise the potential of observing a protective effect. Thus the observed improvement in tubular and glomerular function in RIPC patients could be attributed to effects of RIPC on the ischaemic (RIPC applied on the donor) or reperfusion phase of IR (RIPC applied on the recipient). If the protective effects are confirmed upon completion of the present study, it would be important to determine whether similar protection can be achieved by applying the stimulus on the donor or recipient alone. If RIPC of the recipient results in a similar degree of protection as RIPC on the donor alone or on both donor and recipient, this would allow the use of this potent protective intervention to limit injury in grafts from cadaveric donors (where preconditioning the donor is logistically difficult). Because IR injury is greater in cadaveric transplantation, RIPC might have a greater absolute benefit in this patient group.

One potential limitation of the present study arises from the fact that in all patients that took part in the study the native kidney(s) were left *in situ* when the new kidney

was transplanted. Thus, some of the RBP "leaking" in the urine following transplantation could have originated from the native kidney(s) as a result of the disease process affecting the kidney. It is possible that leak form the native kidney contributes to the urine RBP levels after 72 hours, when concentration remains significantly higher than that reported for healthy children. Nonetheless, the exact contribution of the native kidneys in RBP excretion post-transplantation was not determined; in this respect, determination of the total amount of RBP excreted pre-operatively should provide an estimate and will be performed in some of the patients recruited prior to the completion of the study.

8.5 Conclusions:

These preliminary data suggest that delayed RIPC reduces IR injury following renal transplantation. If confirmed by completion of recruitment, these data support larger scale studies to investigate whether RIPC will have a clinically meaningful effect on tissue injury in patients.

Chapter 9:

Conclusions

9.1 Introduction

This thesis characterised the phenomenon of remote ischaemic preconditioning (RIPC) in humans. The protective potential of RIPC applied on the limb was established in a human model of endothelial ischaemia-reperfusion (IR) injury in the brachial artery of healthy volunteers, and evidence was provided for a graded protective response determined by the "strength" of the preconditioning stimulus. I demonstrated that there are two phases of protection by RIPC; an early relatively short phase active immediately, and a second window of protection that manifests 24 hours following the application of the RIPC stimulus, lasting for up to 48 hours. Both early and late protection by RIPC were dependent on intact autonomic function, supporting a key role of the autonomic nervous system in the transfer of the protective signal from the site of preconditioning to remote tissues. Further insight in the mechanisms of RIPC in humans was provided by studies in which protection by RIPC was blocked by systemic glibenclamide, demonstrating the involvement of ATP-sensitive potassium (K_{ATP}) channels in protection by RIPC. Finally, the protective potential of RIPC against clinical IR injury was investigated in paediatric patients undergoing renal transplantation and preliminary data suggest a beneficial effect of RIPC in this setting.

9.2 Endothelial IR injury in humans

In most studies presented in this thesis IR was induced in the arm of healthy volunteers, by inflating a blood pressure cuff to supra-systolic pressure for 20 minutes, followed by cuff deflation and reperfusion. The latter resulted in conduit vessel endothelial dysfunction at 20 minutes of reperfusion (demonstrated by a reduction in brachial artery flow-mediated dilation), but had no effect on vascular

smooth muscle function. This *in vivo* model of IR was developed by Kharbanda *et al* (244), and results in endothelial injury that recovers 60 minutes following reperfusion. The latter is consistent with IR-induced endothelial stunning. The present human model provides a risk-free and reproducible way to induce quantifiable IR injury, and thus it is useful in assessing the effects of putative protective interventions, such as RIPC, against this type of injury. The procedure was well tolerated by all study participants, allowing repeated experiments to be performed on the same volunteers that facilitated both recruitment and analysis of data.

Data presented in *chapter 7* suggest that, in the IR model used in this thesis, the majority of the injury occurs during the reperfusion phase of IR, as endothelial dysfunction could be prevented by interventions administered during ischaemia (*Acute*RIPC) or immediately at the onset of reperfusion (post-conditioning; PostC). These results are consistent with events occuring during the first moments of reperfusion determining the extent of injury. Although it is likely that prolonging the duration of arm ischaemia would eventually result in some degree of ischaemic injury, this was not attempted to avoid causing severe discomfort and/or permanent damage to healthy volunteers.

9.3 Protection by RIPC against endothelial IR injury in humans

Studies presented in *chapter 3* in this thesis demonstrated that RIPC of the limb could prevent endothelial IR injury in the brachial artery in healthy volunteers, which is in agreement with previous observations from our group in the resistance vasculature of the arm (243). The protective effects of RIPC were reproducible, as RIPC consistently protected against IR-induced endothelial dysfunction in several healthy volunteer

studies presented in subsequent chapters. Moreover, protection by RIPC was found to depend on the "strength" of the preconditioning stimulus, as varying the number of RIPC cycles or the volume of tissue undergoing preconditioning resulted in varying degrees of protection, with evidence of a graded response. Further studies would be required to fully characterise the "dose-response" relationship for RIPC (graded protective response versus preconditioning "threshold"), as studies presented in *chapter 3* were underpowered to detect significant differences in the protective potential of the various RIPC stimuli used.

Studies presented in *chapter 4* examined the temporal characteristics of protection by RIPC. A RIPC stimulus applied immediately before IR resulted in protection that disappeared after 4 hours and reappeared 24 hours following RIPC administration, lasting for up to 48 hours. These results are consistent with early and late protection by RIPC against IR injury in humans and are in agreement with the time-course of protection reported for local ischaemic preconditioning (IPC). Moreover, studies in *chapter 6* established that protection by RIPC was blocked by glibenclamide. These data suggest that RIPC is dependent on opening of ATP-sensitive potassium (K_{ATP}) channels, although the exact channel subtype (sarcolemmal versus mitochondrial) was not determined due to the fact that a non-selective K_{ATP} channel blocker was used. These results revealed striking similarities between local and remote IPC in humans and suggest that they may be facets of the same biological phenomenon. Thus, RIPC applied non-invasively on non-vital tissues may be an appealing approach that could be used to harness the potent protective effects of preconditioning in the clinical arena.

One of the main factors that has, so far, limited the clinical application of preconditioning is the requirement for the preconditioning stimulus to be applied in advance of index ischaemia, which in most instances (e.g. myocardial infarction, stroke) cannot be predicted. In this thesis, I have investigated two potential ways of addressing this limitation. In the first instance, studies in *chapter 4* demonstrated that it may be possible to non-invasively induce continuous protection against IR, by administering RIPC stimuli repeatedly at regular intervals (*Chronic*RIPC). This approach took advantage of the prolonged protection by second window RIPC to abolish the gap in protection between early and late phases of protection, did not result in the development of tachyphylaxis, and could be used to trigger a chronic preconditioned state in vital tissues of at-risk individuals in anticipation of acute ischaemic events. Secondly, the fact that in RIPC the stimulus is applied in a tissue different to the one subjected to IR, allowed me to investigate the protective potential of RIPC against injury occuring during the reperfusion phase of IR, by administering RIPC during index ischaemia (*Acute*RIPC). These studies, presented in *chapter 7*, demonstrated that similar protection can be achieved when RIPC stimuli of sufficient strength are applied either in advance or following the onset of an ischaemic insult. If these observations in the endothelium can be extrapolated to other organs, they suggest that there is potential for RIPC to reduce tissue injury if initiated during ischaemia and before reperfusion, providing an alternative approach that could allow protection by RIPC to be utilised in the acute setting of myocardial infarction or stroke, where it is more likely to provide a clinical benefit.

9.4 Transfer of protection to remote tissues

In *chapter 5*, I investigated the role of the autonomic nervous system (ANS) in the spread of protection by RIPC. Systemic administration of the autonomic blocker trimetaphan camsylate abolished early and late protection by RIPC. This is consistent with a neuronal mechanism involved in the transfer of protection form the site of preconditioning to remote tissues, and is in agreement with evidence from animal studies. Studies in this thesis did not determine how activation of the ANS results in systemic protection, but it is possible that release of RIPC triggers (e.g. adenosine, bradykinin) causes autonomic activation (either directly or via activation of sensory nerves), which subsequently results in protection of remote tissues by K_{ATP} channel activation or other, still unidentified, end-effectors. In the present studies, trimetaphan was administered during the application of the preconditioning stimulus, after which the intravenous infusion was stopped. Trimetaphan has a short duration of action, and as a result it is unlikely that its effects were present during induction of IR. Given the schedule of trimetaphan administration, data presented in *chapter 5*, support a role of the ANS in the trigger limb of the RIPC signal transduction pathway.

9.5 Protection by RIPC against clinical IR injury

Following characterisation of the time-course and mechanisms of RIPC, using the *in vivo* model of endothelial IR injury in humans, I organised a randomised clinical trial aiming to determine whether RIPC is protective against clinically relevant IR injury, in the setting of paediatric renal transplantation. Given that endothelial IR injury is consistent with transient endothelial stunning, rather than necrosis, it is possible that protocol of limb RIPC used might be insufficient to protect from more substantial injury. Nonetheless, results presented in *chapter 8* suggest that RIPC offers protection

against IR injury suffered by the kidney during transplantation. RIPC applied 24 hours prior to transplantation resulted in improved post-operative graft tubular and glomerular function compared to control, consistent with second window protection by RIPC against renal IR injury. The degree of protection in preconditioned patients (approximately 40% reduction in RBP excretion in the urine and in the time required for plasma creatinine to halve) is similar to that achieved by a similar RIPC stimulus in a porcine model of myocardial IR injury (50% reduction in infarct size) (243). Moreover, recent preliminary data in humans indicates that preconditioning using limb ischemia reduces troponin T release from the myocardium following cardiopulmonary bypass in children (91). These observations suggest that the limb is a suitable substrate to trigger systemic preconditioning to protect against clinical IR syndromes and that the model of endothelial IR injury used in healthy volunteer studies in this thesis is suitable to investigate the mechanisms of RIPC in humans, *in vivo*.

9.6 Further work

9.6.1 Mechanism of IR Injury

As discussed in *section 1.2.2.2.2* the burst of oxygen free radicals upon reperfusion has been implicated as a key mediator of injury suffered during reperfusion of post-ischaemic tissues. Sources of reactive oxygen species that have been suggested, include NADPH oxidase in leucocytes (neutrophils) and non-phagocytic tissues. In order to determine the role of ROS produced by NADPH oxidase in the IR injury model in the human arm, I have recently completed a study of the effects of IR on brachial artery endothelial function in patients with Chronic Granulomatous Disease *(CGD)*. CGD patients have molecular disruptions in one of the subunits of NADPH

oxidase, rendering the enzyme inactive (412). These studies were performed in collaboration with the Departments of Paediatric Immunology (Prof. T.W. Kuijpers) and Vascular Medicine (Dr. E. de Groot), Academic Medical Centre, University of Amsterdam, Netherlands. IR did not result in endothelial dysfunction in this patient group (human NADPH oxidase "knock-out" model), which was in contrast to what I observed in age- and sex-matched healthy controls. These observations provide direct evidence for a role of ROS in IR injury in the human vasculature, and implicate NADPH oxidase as a major site for ROS production in this setting. Despite the uncertainties regarding the exact cellular location of NADPH oxidase (neutrophils versus vascular wall), these results, together with data from *Acute*RIPC and PostC studies presented in *chapter 7* in this thesis, suggest that endothelial injury observed in our IR model may be caused by ROS produced by NADPH oxidase during early reperfusion.

9.6.2 Mechanism of RIPC

Despite the central role of the autonomic nervous system in the mechanisms of RIPC to remote tissues, it would be interesting to investigate whether humoral mediators are also involved in the transfer of protection by RIPC to remote tissues. Most animal studies have implicated endogenous opioids as putative humoral mediators of RIPC, but it is not currently known whether opioids are involved in the mechanisms of RIPC in humans. The latter could be determined in healthy volunteer studies, similar to the ones described in this thesis, in which the non-selective opioid inhibitor naloxone is administered prior to the application of the RIPC stimulus.

Moreover, in order to further investigate the role of the autonomic nervous system in the mechanisms of RIPC, I have organised a study to determine whether protection against endothelial IR injury can be induced in patients with Pure Autonomic Failure (PAF; Bradbury-Eggleston syndrome) and Multiple Systems Atrophy (MSA; Shy-Drager syndrome). Both PAF and MSA patients present with primary autonomic failure, but MSA is associated with involvement of the brain and spinal cord (causing Parkinsonism or cerebelar problems). Thus, it may be possible to establish which part of the autonomic nervous system (central versus peripheral) is involved in the mechanisms of protection by RIPC in humans. These studies are performed in collaboration with the Autonomic Unit, National Hospital of Neurology and Neurosurgery (Prof. C.J. Mathias).

9.6.3 Clinical studies

My view is that a robust effect of RIPC on surrogate markers of IR injury needs to be established before planning larger scale studies measuring cardiovascular end-points. In this respect, the clinical study in paediatric renal transplantation described in *chapter 8* is likely to yield important results on the protective potential of RIPC against clinical IR. Studies of similar design may also establish whether RIPC can protect against myocardial IR injury in atherosclerotic patients undergoing exercise testing, dobutamine stress echocardiography, angioplasty or open-heart surgery. Due to the fact that age-related reduction in the effectiveness of preconditioning has been reported (because of age *per se* or the effects of cardiovascular disease) (141;288;424), I performed preliminary studies to determine whether the phenomenon of RIPC remains intact in older patients with confirmed atherosclerosis, the principle group to benefit from tissue protection. These studies demonstrated that IR resulted in

endothelial dysfunction that was prevented by RIPC, establishing that the mechanisms of protection remain intact in these patients. Our group, in collaboration with investigators at the Royal Brompton Hospital, are currently performing a clinical trial aiming to determine the effects of early and late RIPC against IR injury in the setting of coronary artery bypass grafting (CABG) surgery, using serum troponin T as a marker of myocardial injury. This study is a *"proof of principle"* investigation, and its design was based on experimental evidence presented in this thesis.

Appendix A:

Summary of Studies on Remote Ischaemic Preconditioning

Ref	Model	RIPC Ischaemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(161)	Rat	Permanent	1	Immediately before	Heart (LAD Occlusion)	60 minutes	Infarct Size	Not Protective
(161)	Rat	15 minutes	1	Immediately before	Heart (LAD Occlusion)	60 minutes	Infarct Size	Protective (Normothermic/Hypothermic)
(471)	Rabbit	10 minutes	1	Immediately before	Heart (LCA occlusion)	30 minutes	Infarct Size, CK	Protective
(471)	Rabbit	10 minutes	1	24 hours before	Heart (LCA occlusion)	30 minutes	Infarct Size, CK	Protective (2nd window)
(423)	Rat	15 minutes	1	Immediately before	Heart (LCA Occlusion)	60 minutes	Infarct Size	Protective
(510)	Rat	30 minutes	1	24 hours before	Heart (LAD Occlusion)	30 minutes	Infarct Size, MPO activity	Protective (2nd window)
(525)	Rat	4 minutes	6	24, 48, 72 hours before	Heart (LCA Occlusion)	45 minutes	Infarct Size, CK	Protective (2nd window)
(389)	Rat	15 minutes	1	Immediately before	Heart (LCA Occlusion)	40 minutes	NADH Diaphorase, LDH	Protective
(306)	Rat	15 minutes	1	Immediately before	Heart (LCA Occlusion)	60 minutes	Infarct Size	Protective
(306)	Rat	5 minutes	3	Immediately before	Heart (LCA Occlusion)	60 minutes	Infarct Size	Not Protective
(520)	Rat	15 minutes	1	Immediately before	Heart (LCA Occlusion)	30 minutes	Infarct Size	Protective
(511)	Rat	25 minutes	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Protective
(511)	Rat	Permanent	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Protective
(213)	Rat (young)	4 minutes	6	24 hours before	Heart (LCA Occlusion)	45 minutes	Infarct Size, CK	Protective (2nd window)
(213)	Rat (senescent)	4 minutes	6	24 hours before	Heart (LCA Occlusion)	45 minutes	Infarct Size, CK	Not Protective
(378)	Rat	15 minutes	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Protective
(378)	Rat	5 minutes	3	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Protective
(305)	Rat	15 minutes	2	between 60 and 175 minutes before	Coronary Artery Occusion	60 minutes	Infarct Size	Protective

Table A.1 RIPC induction by intestinal ischaemia (mesenteric artery occlusion). Negative studies are in red.

Ref	Model	RIPC Ischaemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(161)	Rat	15 minutes	1	Immediately before	Heart (LAD Occlusion)	60 minutes	Infarct Size	Protective (Hypothermic)
(381)	Rabbit	10 minutes	1	Immediately before	Heart (LCA Occlusion)	30 minutes	Infarct Size	Protective
(467)	Rabbit	10 minutes	1	Immediately before	Heart (LCA Occlusion)	40 minutes	Infarct Size, PCr, ATP, Ph(i)	Protective
(121)	Rabbit	10 minutes	1	Immediately before	Heart (??? Occlusion)	45 minutes	Infarct Size	Protective
(372)	Mice	30 minutes	1	15 days before	Kidney (Contralateral RA Occlusion)	30 minutes	Plasma and Urine Sodium, Creatinine, Blood Urea Nitrogen, GFR	Not Protective
(449)	Rat	5 minutes	4	Immediately before	Coronary Artery Occusion	30 minutes	Infarct Size	Protective

Table A.2 RIPC induction by renal ischaemia. Negative studies are in red.

Ref	Model	RIPC Ischaemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(20)	Rat	10 minutes	1	Immediately before	Kidney (LRA occlusion)	45 minutes	TNFa, TBARS, Histopathology	Protective
(71)	Rat	5 minutes	2		Gastric-celiac artery clamping	30 minutes	Lesion Area & Gastric Blood Flow	Protective
(69)	Rat	5 minutes	2	Immediately before	Celiac Artery Occluion	30 minutes	Lesion Area & Gastric Blood Flow	Protective

Table A.3 RIPC induction by hepatic ischaemia.

Ref	Model	RIPC Ischaemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(37)	Rat	2 hours	1	72 hours before	Brain (Bilateral Middle Cerebral artery occlusion	??	Necrosis of Hippocampal CA1 neurons in contralateral hemisphere	Raised Expression of cFOS, Protective (2nd window)
(405)	Mice	4 minutes	6	24 hours before	Coronary Artery Occusion	45 minutes	Infarct Size	Not Protective
(109)	Pig	10 minutes	1	Immediately before	Heart (LAD Occlusion)	60 minutes	Infarct Size	Not Protective
(109)	Pig	30 minutes	1	Immediately before	Heart (LAD Occlusion)	60 minutes	Infarct Size	Not Protective
(478)	Mice	??	1	24 - 32 hours before	Heart (Inflow Occlusion)	40 minutes	Infarct Size, cTnT	Protective (2nd window)

Table A.4 RIPC induction by cerebral ischaemia. Negative studies are in red.

Ref	Model	RIPC Ischaemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(512)	Rat	5 minutes	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Protective 5/10<10/10<15/10
(512)	Rat	10 minutes	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Protective 5/10<10/10<15/10
(512)	Rat	15 minutes	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Protective 5/10<10/10<15/10
(512)	Rat	Permanent	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Not Protective
(513)	Rat	15 minutes	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Infarct Size	Protective

Table A.5 RIPC induction by infrarenal aortic occlusion. Negative studies are in red.

Ref	Model	RIPC Iscahemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(303)	Dog	5 hours	1	48 hours before	Contralateral Gracilis muscle	5 hours	Size of Necrotic Area	Protective (2nd window)
(49)	Rabbit (FAO)	30 minutes	1	Immediately before	Heart (LCA Occlusion)	30 minutes	Infarct Size	Protective
(389)	Rat (FAO)	30 minutes	1	Immediately before	Heart (LCA Occlusion)	40 minutes	NADH Diaphorase, LDH	Protective
(268)	Rat (FAO)	10 minutes	1	Immediately before	Flap Ischemia	3 hours	Flap necrosis	Protective
(524)	Sheep (IAO)	5 minutes	3	Immediately before	Heart (LAD, Diag., Cx Occlusion)	10 minutes	PVR, PAP, PaO2, PaO2/FiO2	Protective

Table A.6 RIPC induction by iliac/femoral occlusion

Ref	Model	RIPC Iscahemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(368)	Rat (H/L)	10 minutes	1	Immediately before	Heart (LAD Occlusion)	30 minutes	Ventricular Tachyarrhythmia	Protective
(268)	Rat (H/L)	10 minutes	1	Immediately before	Flap Ischemia	3 hours	Flap necrosis	Protective
(267)	Rat (H/L)	10 minutes	1	Immediately before	Flap Ischemia (cremaster muscle)	2 hours	Leukocyte count & RBV	Protective
(6)	Pig (H/L)	10 minutes	3	Immediately before	Latissimus Dorsi muscle	??	Size of Necrotic Area	Protective
(243)	Pig (H/L)	5 minutes	4	Immediately before	Heart (LAD Occlusion)	40 minutes	Infarct size & Ventricular Function (EF)	Protective
(265)	Rat (H/L)	10 minutes	1	Immediately before	Flap ischemia	3 hours	Necrotic Area	Protective
(7)	Pig (H/L)	10 minutes	3	Immediately before	LD, GC, RA muscle flaps	240 minutes	Muscle Flap Infarct Size, ATP depletion time, MPO activity	Protective
(266)	Rat (H/L)	10 minutes	1	24 hours before	Epigastric Flap	180 minutes	Size of Necrotic Area	Not Protective
(266)	Rat (H/L)	10 minutes	1	24 hours before	Cremasteric Flap	120 minutes	Red Blood Cell Velocity in arterioles, venules, capillaries, No of adhering leukocytes in pot cap. Venules	Protective (2nd window)
(342)	Pig (H/L)	10 minutes	3	Immediately before	Pedicle of flap	4 hours	Infarct Size	Protective
(262)	Rat (H/L)	5 minutes	4	Immediately before	Heart (LCA Occlusion)	45 minutes	Infarct Size	Protective
(297)	Mice (H/L)	5 minutes	6	24 hours before	Global	40 minutes	Infarct Size	Protective (2nd window)
(181)	Human (Arm)	3 minutes	2	Immediately before	Heart (Aorta Occlusion)		CPK, CPK-MB, LDH	Protective?
(243)	Human (Arm)	5 minutes	3	Immediately before	Forearm (non-dominant)	20 minutes	Endothelial Function-in response to Ach	Protective
(258)	Human (Arm)	5 minutes	3	N/A	None	–	Neutrophil gene expression	Inflammatory gene downregulation

Table A.7 RIPC induction by limb/hindlimb ischaemia (non-invasive). Negative studies are in red.

Ref	Model	RIPC Ischaemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(398)	Dog	5 minutes	4	Immediately before	Heart (LAD Occlusion)	60 minutes	Infarct Size	Protective
(470)	Rabbit	5 minutes	4	0, 3, 24, 48, 72, 168 hours before	N/A	N/A	Expression of HSP72/73 in non-preconditioned cardiac tissue	Raised Expression of HSP72/73/Protective?
(352)	Rabbit	5 minutes	2	Immediately before	Heart (Global Isch in Vitro)	30 minutes	Infarct Size	Not Protective
(27)	Dog	3 minutes	4	Immediately before	Heart (Aorta Occlusion)	20 minutes	Myocardial Wall Thickness, Glucose Metabolic Rates, Myocyte Contractility, [Ca]i	Protective
(352)	Rabbits	5 minutes	2	Immediately before	Heart (Global Ischaemia)	30 minutes	Infarct Size	Not Protective
(71)	Rat	5 minutes	2	Immediately before	Gastric-celiac artery clamping	30 minutes	Lesion Area & Gastric Blood Flow	Protective

Table A.8 RIPC induction by ischaemia of remote cardiac region. Negative studies are in red.

Ref	Model	Type of RIPC Stimulus	RIPC Ischaemia	Cycle No	RIPC applied	Site of index ischaemia	Ischaemia	End Point	Effect
(303)	Dog	Gracilis muscle arterial supply occlusion	5 hours	1	48 hours	Contralateral Gracilis muscle	5 hours	Size of Necrotic Area	ATP preservation/ HSP expression? Protective
(37)	Rat	Middle Cerebral Artery Occlusion	2 hours	1	72 hours before	Brain	??	Necrosis in contralateral hemisphere	Raised Expression of cFOS, Protective
(470)	Rabbit	Right Coronary Artery Occlusion	5 minutes	4	0, 3, 24, 48, 72, 168 hours before	N/A	N/A	↑ HSP72/73 in non-preconditioned cardiac tissue	Raised Expression of HSP72/73/Protective?
(471)	Rabbit	Mesenteric Artery Occlusion	10 minutes	1	24 hours before	Heart (LCA occlusion)	30 minutes	Infarct Size, CK	Protective
(510)	Rat	Mesenteric Artery Occlusion	30 minutes	1	24 hours before	Heart (LAD Occlusion)	30 minutes	Infarct Size, MPO activity	Protective
(525)	Rat	Mesenteric Artery Occlusion	4 minutes	6	24, 48, 72 hours before	Heart (LCA Occlusion)	45 minutes	Infarct Size, CK	Protective
(372)	Mice	Renal Artery Occlusion	30 minutes	1	15 days before	Kidney (Contralateral RA Occlusion)	30 minutes	Plasma and Urine Sodium, Creatinine, Blood Urea	Not Protective
(478)	Mice	Global Cerebral Ischaemia	??	1	24 - 32 hours before	Heart (Inflow Occlusion)	40 minutes	Infarct Size, cTnT	Protective
(213)	Rat (young)	Mesenteric Artery Occlusion	4 minutes	6	24 hours before	Heart (LCA Occlusion)	45 minutes	Infarct Size, CK	Protective
(213)	Rat (senescent)	Mesenteric Artery Occlusion	4 minutes	6	24 hours before	Heart (LCA Occlusion)	45 minutes	Infarct Size, CK	Not Protective
(266)	Rat	Hind limb	10 minutes	1	24 hours before	Epigastric Flap	180 minutes	Size of Necrotic Area	Not Protective
(266)	Rat	Hind limb	10 minutes	1	24 hours before	Cremasteric Flap	120 minutes	Red Blood Cell Velocity in arterioles, venules,	Protective
(297)	Mice	Hind limb	5 minutes	6	24 hours before	Heart (global ischemia)	40 minutes	Infarct Size	Protective

Table A.9 Studies investigating late protection by RIPC. Negative studies are in red.

Appendix B:

Summary of Studies Investigating the

Role of K_{ATP} Channels in the Mechanisms of Ischaemic Preconditioning

Ref	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(126)	Rats. In vitro.	**Mitochondrial volume, membrane potential, function, + outer membrane permeability to cytC.** Control vs I/R group (30/15 as shown).	30 mins ischaemia, 15 mins reperfusion.	Diaz (100μM) +/- 5-HD (300μM) added to perfusate 20 mins before ischaemia.	Diaz a) delays onset + ↓ amplitude of cont-racture. b) ↓permeability (P) to cytC c) ↑ mitochondrial vol, maintaining low outer P to nucleotides d) preserves ADP
(261)	Rats. In vitro.	**MitoTracker Red (MTR) to detect ROS.** Diaz or Pin given 30mins prior to addition of MTR. 5-HD, Tiron, MPG, Val, Myx, DNP, HMR 1883, Chel were all incubated for 40 mins with MTR +/- Diaz (for 30mins).	None. Assumption that K_{ATP} channels give protection through IPC.	Diaz (200μM) or Pin (100μM) given for 30mins. 5-HD (1mM), Tiron[1] (10mM),MPG[1] (400μM), Val (25nM), Myx (0.2μM), DNP (200μM), HMR 1883 (10μM), Chel (2μM) all incubated with MTR for 40 mins +/- Diaz (200μM) for 30mins, after 10 mins alone.	5-HD blocked the ↑ fluorescence by Diaz/Pin ($p < 0.05$), but not ↑ by Valino-mycin. Myx + DNP + ROS scavengers[1] blocked all 3 agonists. Diaz was blocked also by HMR 1883 but not Chelerythrine Opening of K_{ATP} channels ↑ ROS prod.
(366)	Rats(aortic smooth musc cells) In vitro.	**MitoTracker Red (MTR) to detect ROS.** Cells stained with reduced MTR for 15mins. Control vs Diaz/Val/ACh/ Methacholine fluorescence, and comb-ined with 5-HD/MPG and GEN/WORT	Side exp using isolated rabbit hearts - I/R 30mins/ 180mins.	**Agonists** : Diaz (200μM), Val (25μM), ACh (2mM), Met (2mM). **Antagonists** : *Of ACh* – atropine (100μM) +4-DAMP (1μM) used ind-ividually + with ACh. *Of All* – 5-HD (1mM) + MPG (1mM),&GEN (50μM)+ WORT(10nM)	ACh ↑ fluorescence,blocked by atropine/ 4-DAMP + 5-HD/MPG + GEN/WORT. Val + Diaz also ↑ fluorescence, blocked by 5-HD but ***not*** by 4-DAMP/atropine or pertussis toxin (ribosylates G_i proteins).
(144)	Rats. In vitro.	**Intracellular ROS generation, contr-actile recovery+^{31}P-NMR spectro-scopy.** Adult cardiomyocyte (ACM)+ perfused heart models (PHM) used.	20 mins ischaemia, 20 mins reperfusion.	**ACM** : Effect of Pin (10μM/l), and Diaz (50μM/l) +/- 5-HD (50μM/l), MPG (100μM/l), NAC (4mM/l) of ROS. **PHM** : Effect of Diaz (100μM/l)+/or NAC (4mM/l) given for 10mins prior to the ischaemia.	50μM/l Diaz ↑ ROS generation, blocked by MPG + NAC. 100μM/l Diaz ↑ con-tractile recovery, ↓ intracellular acidif-ication, and ↑ post ischaemic phospho-creatine, all attenuated with NAC.
(507)	Rabbits. In vitro.	**Infarct size.** *Trigger vs Mediator effect* Diaz given for 5mins, 15mins before ischaemia +/- 5-HD, Chel, Nifedipine for 10mins, 5mins before Diaz(**trigger**) . Or 5-HD*2[ ]s or Glib for 35 mins, 5mins before ischaemia (**mediator**).	30 mins ischaemia, 120 mins reperfusion.	**Trigger** : Diaz (100μM), 5-HD(50μM), Chel (5μM), Nifedipine (100nM). **Mediator** : 5-HD(50μM or 200μM), Glib (10μM). **Also,** Diaz (100μM) given for 35mins, 5mins before ischaemia +/- Chel (5μM) for 40mins, 10mins before ischaemia.	Diaz for 10mins ↓ infarct size, blocked by nifedipine (L-type Ca^{2+} blocker), 5-HD, or Chel when given for **trigger**. 50 μM 5-HD nor Chel did not block when given for **mediator** (5-HD 200μM/Glib did), indicating a PKC independent role.
(509)	Rats. In vitro.	**Cardiac function and fluorescence micro-scopy of PKC isoforms.** Control vs I/R vs Diaz vs PKC down-regulation (its effect on K_{ATP} channels) vs direct activation of PKC.	40 mins ischaemia, 30 mins reperfusion.	Diaz (80μM) or PMA (100nM/L-direct activation) for 6 mins prior to I/R. PMA (0.4μg/kg) every 4hrs for 24hrs (down-regulate). Western blots(*) + immunocyto-chemistry(*) to localize diff. PKC isoforms.	Diaz ↑ cardiac function + ↓ cell injury, this was abolished in PKC down-regulated hearts. (*) demonstrated that PKC-α and PKC-δ were translocated to sarcolemma + PKC-δ to mitochondria.
(420)	Rabbits. In vitro.	**Flavoprotein fluorescence and membrane current.** Rabbit ventricular myocytes used to assess sarco K_{ATP} & mito K_{ATP} channels.	60 or 120 mins, by *cell pelleting model.*	Nicorandil (100μmol/L) 15mins before ischaemia, with/without 5-HD (500μmol/L) or HMR 1098 (30μmol/L) – sarcolemmal ***K_{ATP} blocker***.	Nicorandil ↓ rate of cell death compared to control ($p<0.01$). This was blocked by 5-HD but NOT by HMR 1098.

Table B.1.1 Role of K_{ATP} channels in ischaemic preconditioning; Pharmacological preconditioning, *in vitro* studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(294)	Isolated erythrocyte-perfused rat heart model.	**Cardiac function Coronary blood flow conc.**	12 mins mild insult, to cause stunning.	Glib (0.005, 0.05, 0.25, 1,4 umol/l) Glim (0.005, 0.05, 0.15, 0.25, 0.8)	Both Glib & Glim reduced blood flow (conc-dependent). This was more pronounced post-ischaemia. Highest conc of glib reduced dysfunction, therapeutic doses unprotective. Whereas lowest conc of Glim was protective.
(208)	Rats. In vitro	**Mitochondrial membrane potential, matrix volume, Ca^{2+} transport.** These variables measured before and after K_{ATP} openers +/- K_{ATP} blockers.	N/a. Isolated mitochondria, from anaes-thetised rats	Pinacidil (100μM), Cromakalin (25μM), Levcromakalim (20μM), Val (12.5ng/mg), +/- Glyburide (1μM), and 5-HD (20μM).	K_{ATP} openers induced membrane depol-arisation, abolished by 5-HD/Glyburide. Also K_{ATP} openers ↑ Ca^{2+} release (blocked by Gly), and ↑ matrix volume.
(419)	Rabbits. In vitro	**Mitochondrial membrane potential (flavoprotein fluorescence).** Pharmacological K_{ATP} openers given +/- a PKC activator, +/- K_{ATP} blocker.	N/a. Isolated ventricular myocytes.	Diaz (100μM) or Pin (100μM) +/- PMA (100nM) (PKC activator), +/- 5-HD (2mM). Also inactive 4α-phorbol (100nM) given as a control.	Diaz oxidized membrane redox potential, which was accelerated by PMA. This effect was blocked using 5-HD (but only partially blocking Pin – unselective).
(311)	Rabbits. In vitro	**Mitochondrial membrane potential (flavoprotein fluorescence), and sarcolemmal currents.** Four groups – control vs Diaz +/- 5-HD.	N/a. Isolated ventricular myocytes.	Diaz (50μM) given 15mins before pelleting. 5-HD (100μM) given 20mins before pelleting.	Diaz oxidized membrane redox potential, but didn't activate sarcolemmal K_{ATP} channels. Diaz ↓ cell death in simulated ischaemia, blocked by 5-HD.
(457)	Guinea pigs. In vitro	**Inotropic state, Contractile function and MAPD.** Papillary muscles bathed in solutions opposite with cardioplegic hypoxia.	120 mins ischaemia, 60 mins reperfusion	Vehicle vs Nicorandil (1mM) given throughout ischaemia +/- Glib (1μM) 10 mins before & throughout ischaemia. Also +/- dob-utamine (10μM) for 15mins after reperfusion.	Nicorandil ↑ contractile function + ↑ AP shortening (*but protecteion unrelated to MAPD shortening*), enhanced by dobut-amine & completely blocked by Glib.
(456)	Guinea Pig. In vitro	**Contractile Recovery.** The effect of Nicorandil on myocardial stunning induced by cardioplegia, and the effect of blockade with Glib.	120 mins cardioplegia, 60 mins reperfusion.	Nicorandil at varying concentrations (1, 30 and 100μmol/l and 1mmol/l), and in some groups Glib (1μmol/l).	Nicorandil improved contractile recov-ery, and ↓ developed tension (are the –ve inotropic effects of nicorandil responsible?). This was reversed by Glib.
(176)	Rats. In vitro.	**Isolated hearts – contractile function, ATP reserves, coronary blood flow.** Cromakalin and its enantiomers used against control.	25 mins ischaemia and 25mins reperfusion.	Cromakalin (different concentrations all around 1μM) showed stereoselectivity.	Reperfusion contractile function ↑, lactate dehydrogenase release ↓, ATP preserved at 15-25mins ischaemia, with all compounds of Cromakalin.

Table B.1.2 Role of K_{ATP} channels in ischaemic preconditioning; Pharmacological preconditioning, *in vitro* studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(295)	Rats. Ex vivo.	**Coronary Flow & Cardiac Function** (pre+afterload, giving cardiac output). Drugs were given 10mins before global ischaemia for the stated durations.	12 mins (till stunning), 20 mins reperfusion.	Glib (4µg/l for 20min), + Pin (1µg/l for 20min) or Diaz (30µg/l for 30min), also used individually.	Pin/Diaz ↑ coronary flow, blocked by Glib. Glib/Pin/Diaz ↓ cardiac functional *loss* vs control. Glib +/- Diaz/Pin gave additional protection.
(93)	Rabbits. Ex vivo.	**Infarct size and risk area.** ACh, Bradykinin, Morphine, Phenyl-ephrine and Adenosine given for 5 mins, 15 mins before ischaemia. Also MPG + 5-HD given for 15mins, 5mins before agonist.	30 mins ischaemia, 120 mins reperfusion.	**Agonists:** ACh (0.55mmol/L), Bradykinin (0.4 µmol/L), Morphine (0.3µmol/L), Phenyl-ephrine (0.1µmol/L), Adenosine (100µmol/L)..all for 5 mins, see methodology **Antagonists:** MPG (300µmol/L), or 5-HD (200µmol/L), both for 15mins.	Each agonist induced cardioprotection ($p<0.05$), which was blocked by MPG and 5-HD. However, adenosine is the EXCEPTION as neither MPG or 5-HD blocked this response. *A combination of MPG + 5-HD was not used to block.*
(370)	Rabbits Ex vivo.	Memory component with KATP opening? Though only evaluated up till 30 mins. Critical time for mito KATP channel opening. Does inhibition of PKC/TK block protection by diazoxide?	30 mins CAO 2 hours reperfusion.	Diazoxide (10umol/l) Pinacidil (100umol/l) Glibenclamide (5umol/l) Chelerythrine(5umol/l) Genistein (50umol/l) MPG (300), TBAP (7umol/l) Early or late administration.	Pin & Diaz protective after washout. Thus, memory! Early admin of 5-HD abolished protection (late-protective) Glib blocked protection in early phase. MPG & TBAP blocked protection. Chel (early or late) didn't block protection. Genistein late blocked protection. Diazoxide causes activation of p38 MAPK during the ischaemic insult.
(485)	Rabbits Ex vivo	**Infarct size**. Functional recovery. Adenosine enhanced PC (APC) modulated by KATP –before ischaemia or during reperfusion.	30 mins global ischaemia 120 mins reperfusion.	Glib (18uM) 5-HD (200uM) HMR 1883 (50uM) 2 mins before APC and during the 5 mins after, 5mins before ischaemia (I), 2 mins during reperfusion (R)	Infarct size greater with Glib I and R , 5-HD I and partially R. Glib I and R reduced APC mediated functional recovery. 5-HD I or R had no effect. HMR reduced functional recovery but not infarct size. Thus, infarct size reduction modulated by mito KATP primarily during I, sarc during I and R.
(32)	Rabbits. Ex vivo.	**Infarct size.** Rabbits were pretreated with saline or CCPA for 24hrs. 20 mins before ischaemia either Glib/5-HD/placebo given.	30 mins CAO, and 120 mins reperfusion.	CCPA (0.1mg/kg for 24hrs), Glib (0.3mg/kg for 20min), 5-HD (5mg/kg), or placebo solution.	CCPA limited size of infarction ($p<0.01$), which Glib and 5-HD both blocked. Saline pretreated rabbits were not affected by 5-HD or Glib.
(468)	Rats. Ex vivo.	**Infarct size.** Diaz given at 72, 48, 24, 12 hours before index ischaemia. Also Diaz given solely at 24 hours, + Chel or 5-HD given 10mins before Diaz **or** I/R.	30 mins CAO, and 120 mins reperfusion.	Diaz (7mg/kg iv) given at time points mentioned. 5-HD (5mg/kg iv) or Chel (5mg/kg iv) given either 10mins before Diaz (24 hours) or before I/R.	Diaz ↓ infarct size when given 24 hours before I/R (little effect at 12, 48 + 72 hrs) 5-HD and Chel both blocked protection, when given 10mins before Diaz or I/R.

Table B.2.1 Role of K_{ATP} channels in ischaemic preconditioning; Pharmacological preconditioning, *ex vivo* studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Protective Effect
(149)	Rats. Ex vivo.	**Infarct size.** TAN-67 given 1, 12, 24, 48 or 72 hours before I/R. Also TAN-67 given solely at 48 hours with Glib/ BNTX/5-HD given 30/10/5 mins before I/R (+ BNTX 30mins before TAN-67).	30 mins CAO, and 120 mins reperfusion.	TAN-67 (10 or 30mg/kg) given as indicated. TAN-67 (30mg/kg) given solely with blockers (Glib (1mg/kg), BNTX (3mg/kg), or 5-HD (10mg/kg) prior to I/R. BNTX (6mg/kg) also given 30mins prior to TAN-67 (30mg/kg).	TAN-67 produced maximal protection with 30mg/kg at 48hrs (also significant at 24hrs, none others). BNTX (in both conditions), Glib, and 5-HD all blocked this protection.
(157)	Rats. Ex vivo	**Contractile function, coronary flow and LDH release were measured.** Vehicle vs Cromakalin vs Diaz given for 10mins before index ischaemia.	25 mins (till infarction), 30 mins reperfusion.	Cromakalin (1-100μM), Diaz (1-100μM). EC_{25} (cardioprotective potency). Also Diaz (30μM) (+/-5-HD 100μM) and Cromakalin (30μM), both +/- Glib (0.3μM), for 10 mins.	Diaz+Cromakalin ↑ time of onset to contracture, & ↑ functional recovery (blocked by Glib and 5-HD). Diaz less potent than Crom at ↓ sarc K_{ATP} currents.
(157)	Rabbits Ex vivo	**As above.** As a second part to the above study the authors used a similar model in rabbits.	50 mins ischaemia, 30 mins reperf.	Vehicle vs Diaz (30μM) and Cromakalin (30μM) +/- 5-HD (100μM), again for 10 mins.	Rabbits ↑ resistance to global ischaemia. 'mitoK_{ATP} channels~ 1000* more sensitive to Diaz than sarcK_{ATP} channels'
(441)	Guinea pigs. Ex vivo	**MAPD, developed tension, contractile function.** 2 protocols used, with index ischaemia of 10 and 20 mins respectively, +/- Glib (1) or Cromak (2)	10 mins or 20 mins ischaemia, 60mins reperfusion.	Glib (10μM), used for 20mins before the 10 mins index ischaemia. Cromakalin (1, 2, and 5μM), for 20mins before 20mins index ischaemia.	Glib attenuated action potential shortening, and suppressed contractile function. Conversely, cromakalin ↑ AP shortening + ↑ contractile function (but ↑ VF!).
(331)	Guinea Pig Ex vivo.	Preservation of high energy phosphates. Shortening of action potential duration (APD) & contractile failure.	20 mins global no-reflow with glib, 30 mins with pinacidil.	Glib (10 or 50 μM)	Pinacidil ↑APD shortening & early contractile failure, but inhibited high energy phosphate depletion. Glib inhibited these effects. Glib alone inhibited the early decline in APD & ↑ ischemic contracture, but did not ↑ ATP or CP depletion. Higher conc. of Glib produced greater inhibitions.

Table B.2.2 Role of K_{ATP} channels in ischaemic preconditioning; Pharmacological preconditioning, *ex vivo* studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(279)	Sheep. In vivo.	**% recovery of wall, + monophasic action potential duration (MAPD).** 12mins of ischaemia was preceded with the drugs shown opposite.	12 mins (till stunning), 120 mins reperfusion.	Glib (4mg/kg) +/- Diltiazem (Ca^{2+} channel blocker - 100µg/kg for 20min) infused 30mins before ischaemia.	APD ↓ in control ischaemia and with Diltiazem, but lengthened (with ↑ stunning) with Glib ($p<0.01$). The combination interestingly ↑ cardiac recovery.
(215)	Dogs. In vivo.	**Cardiac function + myocardial segment shortening.** LV catheter + ECG + blood glucose measured. Absence of ↑ stunning with new hypoglycaemic drug.	15 mins of ischaemia, 240 mins reperfusion.	KAD-1229 (0.003/ 0.03/ 0.3/ 3mg/kg), Glib (0.01/ 0.1/ 1mg/kg) or vehicle added 10mins before start of ischaemia.	Recovery from the ↓ % segment shortening was worse with Glib, not with KAD-1229. KAD-1229 has ↑ specificity for pancreatic (not heart) sulfonyl receptors?
(111)	Sheep In vivo	Does Glib elicit protection on postischaemic myocardial stunning and against reperfusion induced **arrhythmias (ASI), systolic wall thickening fraction (%WTH), diastolic compliance (CR)**	12 mins 120 mins reperfusion	Glib (0.4mg/kg) infused for 10 & 30 mins before ischaemia.	Glib increased ASI, reduced %WTH and CR. Also proarrhythmic, VT, VF increased. Mortality increased but not significantly. Glib abolished action potential duration (APD) shortening elicited by ischaemia.
(479)	Dogs In vivo.	**Myocardial infarct size**. Anaesthetic induced preconditioning. Desflurane reduces MI size by activation of specific sarcolemmal/ mito KATP channels?	60 mins LAD occlusion 3 hours reperfusion.	Glyburide (0.1mg/kg) HRR (1mg/kg/min) 5-HD (150ug/kg/min) Given over 45-10mins before, during and 5 mins after desflurane.	Desflurane reduces infaract size compared to control: 25% to 10%. All three blocked the protective effects of desflurane.
(145)	Rats. In vivo	**Myocardial LV work, glycolysis, + proton production.** Hearts were untreated or given Cromakalin/ Glib/CHA or Glib and CHA.	30 mins (till infarction), 30 mins reperfusion.	Cromakalin (10µM), Glib (30µM), CHA (0.5µM), given 5 mins before index ischaemia.	Glib ↑ *glycolysis + proton production*, CHA ↓ both (still ↓ with Glib), Cromakalin no effect. *LV work*: ↑ by CHA, ↓ by Glib, no effect Cromakalin.
(382)	Rabbits In vivo.	**Infarct size**. Role of KATP channels in delayed heat stress induced cardioprotection.	30 mins of ischaemia, 120 mins reperfusion.	Glib (0.3mg/kg) 5-HD (5mg/kg) 10 mins before induction of MI.	Infarct size reduced in heat-stress treated animals. This was abolished by 5-HD and Glib.
(102)	Dogs. In vivo.	**Action potential duration at the 95% repolarisation level (APD95).** APD95 taken pre/post drug, during ischaemia & reperf.	15 mins ischaemia, 30mins reperfusion.	Cromakalim (1mg/kg/min) given before 15mins ischaemia. Glyburide (3mg/kg/min) given to some animals in addition.	Cromakalim ↓ APD95 by 27% compared to 8% with controls. Glyburide abolished APD95 effects. Greater survival rate with cromakalim.

Table B.3 Role of K_{ATP} channels in ischaemic preconditioning; Pharmacological preconditioning, *in vivo* studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(112)	Diabetic sheep model. In vivo.	**% recovery of wall, + monophasic action potential duration (MAPD).** Early[1] (E - before ischaemia) + Late (L - 24hrs before ischaemia) IPC in sheep (normal + diabetic).	12 mins (till stunning), 120 mins reperfusion.	[1]*IPC applied as 6C of 5min ischaemia, and 5 mins reperfusion.* Glib (0.4mg/kg) and (0.1mg/kg) given 30 mins before ischaemia.	E + L IPC protected against stunning in normal sheep, whereas in diabetics, E exaggerated stunning + L had no effect. Diabetic sheep were inhibited by a lower concentration of Glib than normal sheep.
(426)	Pigs. Ex vivo.	**Infarct size.** IPC (1C 10mins CAO + 15min reperfusion) +/- Glib at 2 concentrations.	90 mins (till infarction), 120 mins reperfusion.	i.v. Glib (0.5mg/kg) and (1.5mg/kg), either given 10mins before, and immediately following IPC.	IPC provided protection, which was blocked by Glib given before IPC, but NOT when given post IPC, despite ↑ [ ].
(120)	Rats. In vitro.	**Recovery of contractile force.** *Control vs Normoxic* (50mins perfusion) *vs PC* 5C 5min ischaemia+10mins reperfusion , +/- Glib and Naloxone (opioid antag).	60 mins 'simulated' ischaemia, 30 mins reperf.	Glib (5μM) or Naloxone (5μM) was added 5 mins before the addition of the normoxic PC concentrate.	Recovery ↑ with PC concentrate, attenuated by Glib and Naloxone, therefore *"cardiac effluent protects mesenteric tissue, through opioid + K_{ATP} channels".*
(110)	Diabetic sheep model. In vivo.	**% recovery of wall.** Same E^1 + L^1 IPC in normal/diabetic sheep. Also glucose/insulin given prior E^1 + L^1 IPC in normal/diabetic sheep respectively.Diaz,Glib,5-HD also given	12 mins (till stunning), 120 mins reperfusion.	Mins before ischaemia : 80mins - Diaz (10μg/kg), 30mins Glib (0.4mg/kg) and (0.1mg/kg), 10mins 5-HD (5mg/kg. Glucose (0.5g/kg) or Insulin (10U then 20U bd) for normal and diabetic sheep respectively.	See reference 1 for primary results. Insulin REVERTED the LACK of E + L IPC in diabetics.
(195)	Rats. In vitro.	**Infarct size and MitoPTP opening.** Control vs IPC-2C 5min I/R, vs CsA FK506/Atr (all given at reperfusion), pre treatment with Diaz/CCPA, IPC+Atr, Diaz+Atr, CCPA+Atr.	35 mins ischaemia, 120 mins reperfusion.	CsA (0.2μM/L), FK506 (5μM/L), or Atr (20μM/L) given at reperfusion. Diaz (30μM/L), or CCPA (200nM/L) given before index ischaemia. The effect of K_{ATP} channels on calcein-loaded mitochondria was assessed.	IPC/CsA or pretreatment with Diaz/ CCPA ↓ infarct size, although this was blocked with Atr (& 5-HD abolished Diaz+IPC). Atr/FK506 at reperfusion had no effect. Diaz ↓Ca^{2+} MPTP opening
(461)	KO mice. In vivo & in vitro.	**Infarct size, MAPD+contractile recovery.** Kir6.2(KO) deficient mice (sarco K_{ATP} deficient) vs WT mice. The effect of DNP but glucose free solution (DNPgfs)/IPC*/HMR1098/5-HD noted	I/R diff mins for vivo (45/ 120mins) and vitro (20/60).	**Vivo** : IPC* given as 3C of CAO I/R 3/5min & the effect of DNPgfs (50μM) noted. **Vitro** : HMR-1098 (30μM), Glib (10μM), Diaz (100μM) given to KO+WT mice 15mins prior to ischaemia to assess sarco/mitoK_{ATP}channels	IPC ↓ infarct size in WT not KO mice. DNPgfs ↓ the APD in WT not KO mice. HMR-1098, but not 5-HD, ↓ contractile recovery in WT mice. Diaz induced flavoprotein fluorescence in WT + KO.
(336)	Rats. Ex vivo.	**Infarct size.** 9 studies (IRI +/- a) pre Diaz/ Glim/Glib, b) pre Diaz & Glim/Glib, c) IPC 2C 5min +/- d) Glim/Glib.	35 mins ischaemia, 120 mins reperfusion.	Glib (10μg/l for 20min) or Glim (10μg/l for 20min) & Diaz (30μg/l for 30min).	Glib blocks IPC and Diaz protection ($p<0.05$), Glim does not. ?Glim has no effect on mitochondrial K_{ATP} channels? No effect of Glib/Glim alone.

Table B.4.1 Role of K_{ATP} channels in ischaemic preconditioning; Animal ischaemic preconditioning studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(272)	Rats. In vitro.	**Contractile amplitude, recovery, and mitochondrial function** (premeabilised fibre technique) Control vs I/R vs IPC (CAO I/R 5/5min).	30 mins ischaemia, 15 mins reperfusion.	ADP (1mM) + cytochrome c (8μM) added to assess outer mito membrane integrity. Mito creatine kinase + ADP functional coupling also assessed ADP +/-creatine kinase (20mM)	IPC ↑ contractile recovery, delayed onset of amplitude of contraction + maintained functional coupling between creatine kinase + ADP, and high $K_{1/2}$ for ADP.
(536)	Chicks cardio-myocytes. In vitro.	**Intracellular ROS generation and % cell death.** IPC (I/R 10/10mins) vs Flu for 10mins, 20mins before ischaemia. Flu mimics IPC through K_{ATP} channels?	60 mins ischaemia, 180 mins reperfusion.	Flumazenil (10μM) +/- PKC inhibitors - Chel (2μM) + Go-6976 (0.1μM), or 5-HD (1mM) given throughout I/R. Also MPG (800μM)/ PMA(0.2μM) prior to/during I/R respectively.	IPC, Flu and PMA ↑ ROS and ↓ % cell death similarly. This was abolished with pre-MPG or Chel/Go-6976 during I/R. 5-HD abolished protection by PMA or Flu
(466)	Rabbits In vitro	**Infarction and myocardial stunning (MS)** KATP channels in late phase: end-effectors? IPC (6 Occlusion-Reperfusion cycles for 3 consecutive days - ? effect changes with time?	30 mins CAO. 72 hours reperfusion	5-HD (5mg/kg) given 5 mins before occlusion. 5-HD & Glib (0.3mg/kg) given on day 2. Diazoxide (10ug/kg/min) given on day 1& on day 2 during PC	5-HD 5 mins before index ischaemia blocked protection by IPC (↓ by Diaz), 5-HD/Glib on day 2 didn't. Diaz gave further attenuation when given on day 2. IPC had >effect on MS on days 2 and 3.
(44)	Rabbits. Ex vivo.	**Infarct size.** IPC given as 4C 5mins + 10mins reperfusion, 24 hours before index ischaemia. Glib or 5-HD given. MAPD(deploarisation of K_{ATP} channel)	30 mins CAO, and 180 mins reperfusion.	I/R group vs IPC group. Both groups given Glib (0.3mg/kg iv) 30mins or 5-HD (5mg/kg) 15mins before index ischaemia.	Glib or 5-HD alone didn't affect infarct size. IPC ↓ infarct size ($p<0.01$), + MAPD shortened during index ischaemia which *was* blocked by Glib and 5-HD.
(260)	Rabbits In vivo.	**Infarct Size**. Calcium PC. 2 cycles of 5 mins infusion with 15 mins interval.	30 mins CAO, 180 mins reperfusion	Cromakalin (40ug/kg) pretreatment 10 mins before ischaemia. Glib (1mg/kg) – given 20 mins or 45 mins before ischaemia.	Cromakalin ↓ infarct size. Glib blocked protection by IPC and CPC 45 mins but NOT 20 mins before sustained ischaemia. Verapamil ↓ protection.
(321)	Guinea Pig Ex vivo	Coronary overflow produced by bolus of ACh. SNP used as a measure of endothelium-independent vascular function.	40 mins global ischaemia 40 mins reperfusion.	Pinacidl (170nM) SPT (5uM)-Adenosine receptor antagonist. DPCPX (0.5uM)-A1 antagonist. Adenosine (10uM) CHA (0.25uM)-A1 agonist.Glib (0.6uM)	SPT & DPCPX ↓ ACh response. Glib reduced vasodilatory response of ACh & SNP. Protective effect of IPC, CHA & pinacidil abolished by Glib. IPC not affected by DPCPX or SPT.
(381)	Rabbits. Ex vivo	**Infarct size.** Sham 20mins anaesthesia vs 1C 10min IPC (renal artery occ-lusion), 10min reperfusion. +/- 8-SPT or 5-HD.	30 mins (till infarction), 120 mins reperfusion.	8-SPT (7.5mg/kg) (non-selective adenosine antagonist) or 5-HD (5mg/kg) given 10 or 15 mins respectively before sham/IPC.	IPC ↓ infarct-to-risk ratio vs control. This protection was blocked by both 8-SPT and 5-HD (these agents did not effect sham controls).

Table B.4.2 Role of K_{ATP} channels in ischaemic preconditioning; Animal ischaemic preconditioning studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(400)	Dogs. Ex vivo.	**Infarct size.** a) ACh at 40 mins and 5mins before IRI, b) Nitro-glycerin at same times. c) 4cycles 5mins CAO with 5mins reperf.	60 mins (till infarction), 300 mins reperfusion.	ACh 0.01mg and Nitroglycerin 0.05mg at 40 mins and 5mins before index ischaemia.	Infarct size was ↓ in IPC ($p<0.01$) and ACh ($p<0.05$) groups compared with controls.
(428)	Pigs. Ex vivo	**Infarct size.** Effect of 10 min no-flow ischaemia before index ischaemia is protective, and abolished by Glib.	90 mins ischaemia, 120 mins reperfusion.	Glib (0.5mg/kg iv) then followed by continuous infusion of 50 μg/min, before 10min no-flow ischaemia.	10 mins no flow ischaemia ↓ infarct size (despite lower subendocardial blood flow), which was abolished by Glib.
(430)	Pigs. In vivo	**MAPD, Haemodynamics and Trans-mural Adenosine (ADO) uptake.** The effect on the above of a single preconditioning episode, +/- Glib.	90 mins ischaemia, 120 mins reperfusion.	Local IPC (1C of 10 mins ischaemia, 15 mins reperfusion) with/without Glib (0.5mg/kg iv bolus for 5 mins, followed by infusion 50μg/min for index ischaemia).	MAPD was reduced in the 1st 10 mins index ischaemia>10 mins IPC. Glib abolished this without effecting ADO uptake.
(21)	Dogs. Ex vivo.	**Infarct size.** 5 min CAO with 5 mins reperfusion before prolonged ischaemia.	60 mins (till infarction), 300 mins reperfusion.	5-HD (3μg/kg/min for 20mins, 15mins before index ischaemia) or Glib (3μg/kg/min for 35min) in 5mins IPC or first 5mins index ischaemia.	5-HD or Glib completely blocked the protection from IPC. Neither affected infarct size without IPC.
(173)	Dogs. Ex vivo.	**Infarct size.** 5 min CAO with 10 mins reperfusion before prolonged ischaemia.	60 mins (till infarction), 300 mins reperfusion.	Glib (0.3mg/kg/min for 35min) or placebo a) prior to IPC or b) post IPC. Also RP 52891 (0.1μg/mg/min) infusion before index ischaemia.	Glib did not effect infarct size in non-IPC groups, but in IPC groups Glib ↑ infarct size. RP 52891 also ↓ infarct size ($p<0.05$).
(177)	Dogs. Ex vivo.	**Infarct size.** 5 min CAO with 10 mins reperfusion before prolonged ischaemia, or sham IPC.	60 mins (till infarction), 300 mins reperfusion.	Glyburide (5μg/kg/min) or placebo solution given 20 mins before IPC. Also effect of R-PIA (0.4μg/kg/min) with and without Glyburide noted.	IPC and R-PIA reduced infarct size($p<0.05$). These protective effects were abolished by Glyburide. Glyburide had no effect on infarct size alone.

Table B.4.3 Role of K_{ATP} channels in ischaemic preconditioning; Animal ischaemic preconditioning studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(67)	Human study. In vivo	**Plethysmography**. 6 studies (IRI +/- a) pre D, b) pre Diaz & Glib, c) RIPC ARM 3C 5min +/- d) pre Glib, e) per Glib).	20 mins forearm ischaemia, 15 mins reperfusion.	Glib (10μg/min for 35min) & Diaz (800μg/min for 20min). (? Does ACh give artefact protection)	Glib blocks IPC ($p<0.05$ pre&per) so ↑ chance K_{ATP} channel as effector. Diaz shows mitochondrial K_{ATP} subtype. No effect of Glib alone. *Time course suggests K_{ATP} as effector not trigger.*
(2)	Human study. In vivo	**'Cardiac Endpoint Events.'** 1° versus 2° endpoints. Patients were randomly assigned nicorandil or placebo.	20 mins fore-arm, 15 mins reperfusion.	Nicorandil 10mg bd for 2 weeks then 20mg bd. Patients were followed up for 1-3 years.	Nicorandil ↑ proportion patients event free ($p<0.05$) for 1° endpoint, but significance *depends on clinical category.*
(80)	Human atrial derived cardiomyo-cytes.	**Membrane potential and ROS generation** (by flow cytometry), **mass and matrix volume changes** (by Winlist software) **measured.** *After the simulated ischaemia was 1hr reoxygenation*	LSI – 6hrs incubation in hypoxic buffer, from Esumi *et al** (see footer).	Interventions either '*early*' 30 mins or '*late*' 15mins prior to LSI. Diaz (30μM), 5-HD (50μM), (MPG) (400μM), or NAC (20mM) …both free radical scavengers.	Diaz ↑ oxidation of ROS probe ($p<0.01$), blocked by 5-HD and MPG with no change in membrane potential. Diaz ↑ mitochondrial volume, although no agents affected mitochondrial mass.
(310)	Human embryonic kidney cells.	**Pharmacological comparison of mito K_{ATP} channels to surface K_{ATP} channels.** K_{ATP} channel openers and blockers used to probe character of 6 heterologously expressed K_{ATP} channels	Not applicable	**K_{ATP} channel; openers** : Diaz (100μM), Pin (100μM), or P-1075 (100μM). **blockers** : Glib (10μM), 5-HD (200μM) or HMR-1098 (10μM). ** mK_{ATP} channels closely resemble the Kir 6.1/SUR1 sK_{ATP} channel.	Diaz/Pin open mK_{ATP} channels (but Diaz did not open sK_{ATP} channels), P-1075 does not (but did open all but 1 sK_{ATP} channel). 5-HD but not HMR-1098 blocks the mK_{ATP} channel. Conclusion**
(159)	Diabetic humans undergoing angioplasty	**Death, arrhythmias, complications.** Diabetic patients with acute MI taking sulponylurea vs not taking sulphonyl-urea.	Standard balloon angioplasty protocol.	Group 1. Oral sulphonylurea hypoglycaemics. Group 2. Insulin or controlled by other means.	Sulphonylurea use not assoc with ↑ adverse late events, but IS assoc with ↑ mortality, due to ↓ myocardial tolerance to ischaemia and reperfusion injury.
(50)	Human In vivo	Ischaemic threshold, max ST depression equivalent, wall motion dysfunction. Attenuation of myocardial ischaemia observed with re-exercise- 2 successive Naughton protocol exercise ECG tests.	N/A	Type 2 diabetics used in the study took >10mg daily Glib.	Attenuation observed in all groups. Therefore, independent of intensity of exercise protocol and similar in diabetic subjects. Thus, 'phenomenon does not appear to be mediated by KATP activation.

Table B.5.1 Role of K_{ATP} channels in ischaemic preconditioning; Human studies

Ref.	Animal & Study Type	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Protective Effect
(48)	Human study. In vivo	**Plethysmography.** 6 studies (IRI + Diaz. Then +/- a) Glib, b) Glim or c) Placebo.	60 mins (till infarction), 300 mins reperfusion.	Vasodilator response to Diaz with either Glib (3.3µg/min) or Glim (2.5µg/min) or placebo. (? Does ACh give artefact protection)	Glib blocks Diaz ↑ in forearm vasodilatation ($p<0.01$), whereas neither Glim or placebo did.
(480)	Human study. In vivo	**ST segment voltage marker.** Patients undergoing coronary angioplasty subjected to a) Glib or b) Placebo.	Not applicable. Interventional cardiology.	Glib or Placebo (10mg po) 30 mins before angioplasty. Effects observed between 1st and 2nd balloon inflation (each a 2min inflation followed by 5mins deflation).	Ischaemia during 2nd is < than 1st inflation. This protective effect is blocked by Glib ($p<0.05$), which also caused ↑ in pain with the 2nd inflation.
(480)	Humans undergoing angioplasty	**ST-segment shifts.** 20 patients undergoing one vessel angioplasty given Glib vs placebo, before 2 IPC episodes using balloon inflation.	Standard balloon angioplasty protocol.	Glib 10mg po or placebo given prior to 2 cycles of 2min balloon inflation with 5 minutes reperfusion.	In placebo patients, the ST segment shift in 2nd inflation was less than that of the 1st, whereas there was no change between 1st & 2nd with Glib.

Table B.5.2 Role of K_{ATP} channels in ischaemic preconditioning; Human studies

Ref.	Model	Methodology	Index Ischaemia	Agents used investigating K_{ATP} channels.	Effect
(262)	Rats. Ex vivo.	**Infarct size.** RIPC (4C hindlimb 5min, 5min reperfusion) +/- Glib, 5-HD + HMR 1098 **vs** Local IPC (4C CAO 2min, 3min reperfusion).	45 mins (till infarction), 120 mins reperfusion.	**Antagonists :** Glib (10μM), 5-HD (100μM), and HMR1098 (30μM) before index ischaemia. Diaz (10mg/kg) also given separately.	No effect of antagonists on infarct size in controls. RIPC ↓ infarct size, blocked by 5-HD + Glib, but NOT HMR 1098. IPC and Diaz ↓ infarct size as RIPC.
(342)	Pigs. Ex vivo.	**Infarct size.** RIPC (3C hindlimb 10min, 10min reperfusion) +/- Glib or 5-HD (either 10mins before or at end of RIPC). +/- HMR 1098.	4 hours (till infarction), 48 hours reperfusion.	**Antagonists :** Glib (0.3mg/kg), 5-HD (5mg/kg), and HMR1098 (3mg/kg) 10 mins before RIPC, +/- Glib (1mg/kg) + 5-HD (10 mg /kg) at end RIPC. BMS-191095 (2mg/kg).	RIPC ↓ infarct size, blocked by pre + post RIPC 5-HD + Glib ($p<0.05$), but NOT HMR 1098. BMS-191095 also ↓ infarct size,associated with ATP sparing
(511)	Rats. Ex vivo.	**Infarct size.** Sham vs RIPC(mesenteric I/R 25/15mins). 5-HD, Staurosporine & Chel (pre-ischaemia), + pre-RIPC Hexamethonium used to inhibit.	30 mins (till infarction), 180 mins re-perfusion.	5-HD (5mg/kg), Staurosporine (50μ/kg), Chel (5mg/kg) given 5 mins before the CAO. Hexamethonium (20mg/kg) given 15mins before the RIPC.	RIPC ↓ infarct size vs Sham. PKC + mitoK_{ATP} channel blockers inhibited this, unlike Hexamethonium,suggesting RIPC works through a humoral mechanism.

Table B.6 Role of K_{ATP} channels in ischaemic preconditioning; Remote ischaemic preconditioning studies

Appendix C:

Ethics Committee Approval Documents

Institute of Child Health

and Great Ormond Street Hospital for Children NHS Trust

UNIVERSITY COLLEGE LONDON

30 Guilford Street, London, WC1N 1EH. Telephone: 020 7242 9789 Fax: 020 7905 2201

The child first and always

8 April 2003

Mr S Loukogeorgakis
Vascular Physiology
GOS

Dear Mr Loukogeorgakis,

99CC28 Characterisations of the role of neutrophils in ischaemia reperfusion injury in the human forearm

Thank you for your letter dated 20 March 2003. The Chairman of the Research Ethics Committee, Professor Michael Preece, has on behalf of the Committee approved the re-activation of the FMD part of the above study. He also approved the minor changes to study personnel.

Ethics approval will expire on **31/03/2004** so please inform us if you wish to continue beyond this date.

The decision will be ratified at the full Committee meeting that will take place on Wednesday 7 May 2003 (Please note that you will not receive a letter confirming the above ratification).

Yours sincerely

Administrator to the Research Ethics Committee
020 7905 2620

Great Ormond Street Hospital for Children NHS Trust / The Institute of Child Health Local Research Ethics Committee

Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Tel: 020 7905 2620
Fax: 020 7905 2201
Email: l.howe@ich.ucl.ac.uk

12th November 2003

Mr S Loukogeorgakis
Vascular Physiology
ICH

Dear Mr Loukogeorgakis,

Title: **Characterisation of the effects of ischaemia and reperfusion on endothelial function in humans**
R&D registration number: **99CC17**
Protocol number/version: **N/A**

Thank you for your letter dated 5th November 2003. The Chairman of the Research Ethics Committee, has on behalf of the Committee, approved the amendments as detailed in your letter and summarised below:

- The number of cycles used for the preconditioning stimulus can be varied.
- The original remote ischaemic preconditioning stimulus (3 cycles of 5 minutes of ischaemia followed by 5 minutes of reperfusion) can be applied to the leg instead of the arm.
- The above studies will be performed by Miss Anna Panagiotidou.

The decision will be ratified at the full Committee meeting that will take place on Wednesday 3rd December 2003 (Please note that you will not receive a letter confirming the above ratification).

Yours sincerely

Research Ethics Coordinator

Great Ormond Street Hospital for Children NHS Trust / The Institute of Child Health Local Research Ethics Committee

Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Tel: 020 7905 2620
Fax: 020 7905 2201
Email: l.howe@ich.ucl.ac.uk

22nd December 2003

Mr S Loukogeorgakis
Vascular Physiology
ICH

Dear Mr Loukogeorgakis,

Title: **Role of the autonomic nervous system in remote ischaemic preconditioning in humans**
R&D registration number: **03CC20**
Protocol number/version: **N/A**

Notification of ethical approval

The above research has been given ethical approval after review by the Great Ormond Street Hospital for Children NHS Trust / Institute of Child Health Research Ethics Committee subject to the following conditions.

1. Your research must commence within twelve months of the date of this letter and ethical approval is given for a period of eight months from the commencement of the project. If you wish to start the research more than twelve months from the date of this letter or extend the duration of your approval you should seek Chairman's approval.
2. You must seek Chairman's approval for proposed amendments to the research for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature, eg. using the same procedure(s) or medicinal product(s). Each research project is reviewed separately and if there are significant changes to the research protocol, for example in response to a grant giving body's requirements you should seek confirmation of continued ethical approval.

3. Researchers are reminded that REC approval does not imply approval by the GOS Trust. Researchers should confirm with the R&D office that all necessary permissions have been obtained before proceeding.
4. It is your responsibility to notify the Committee immediately of any information which would raise questions about the safety and continued conduct of the research.
5. On completion of the research, you must submit a report of your findings to the Research Ethics Committee. You may also be required to submit annual reports.
6. Specific conditions pertaining to the approval of this project are:

- The use of the enclosed standard consent forms for the research. A copy of the signed consent form must be placed in the patient's clinical records and a copy must be kept by you with the research records as our insurers may demand access to them.

Yours sincerely

Research Ethics Coordinator

NHS

Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee

The Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Telephone: 020 7905 2620
Facsimile: 020 7905 2201

Email: t.austin@ich.ucl.ac.uk

25 January 2005

Mr Stavros P. Loukogeorgakis
PhD Research Fellow
Institute of Child Health, UCL
Vascular Physiology Unit
34 Great Ormond Stret
London
WC1N 3JE

Dear Mr Loukogeorgakis

Full title of study:	*Effect of blockade of the ATP-sensitive potassium channel on the protective effects of remote ischaemic preconditioning against endothelial ischaemia-reperfusion injury in humans.*
REC reference number:	*05/Q0508/7*
Protocol number:	*Version 1*

Thank you for your letter of 20 January 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair at a meeting on 25 January 2005.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed that they have no objection.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type:	Version:	Dated:	Date Received:
Application		13/12/2004	13/12/2004
Application	Healthy Volunteer Information Sheet, Protocols 1 & 2, v1	13/12/2004	13/12/2004
Investigator CV	version 2	13/12/2004	13/12/2004
Protocol	version 1	13/12/2004	13/12/2004
Covering Letter		13/12/2004	13/12/2004
Letters of Invitation to Participants	Healthy Volunteer Invitation Letter	13/12/2004	13/12/2004
Participant Information Sheet	Healthy volunteer information sheet, version 2	19/01/2005	20/01/2005
Participant Information Sheet	Healthy volunteer information sheet, protocol 3&4, version 2	19/01/2005	20/01/2005
Participant Information Sheet	Healthy volunteer information sheet, protocols 5 & 6, version 2	19/01/2005	20/01/2005
Participant Information Sheet	Healthy Volunteer Information Sheet, Protocols 3 & 4, v1	13/12/2004	13/12/2004
Participant Information Sheet	Healthy Volunteers Information Sheet, Protocols 5 & 6, v1	13/12/2004	13/12/2004
Participant Consent Form	version 1	13/12/2004	13/12/2004
Participant Consent Form	version 2	19/01/2005	20/01/2005
Response to Request for Further Information	Further information as requested by the Committee	20/01/2005	20/01/2005

Management approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Notification of other bodies

The Committee Administrator will notify the research sponsor that the study has a favourable ethical opinion.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q0508/7	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely

Research Ethics Committee

Enclosures *Standard approval conditions*

Site approval form (SF1)

Institute of Child Health

and Great Ormond Street Hospital for Children NHS Trust

UNIVERSITY COLLEGE LONDON

30 Guilford Street, London, WC1N 1EH. Telephone: 020 7242 9789 Fax: 020 7905 2201

The child first and always

30th July 2003

Mr S Loukogeorgakis
Cardiothoracic Unit
ICH

Dear Mr Loukogeorgakis,

Title: **Effects of remote ischaemic preconditioning on ischaemia reperfusion injury in the human kidney during paediatric renal transplantation**

R&D registration number: **03CC11**

Protocol number/version: **N/A**

Notification of ethical approval

The pilot study phase of the above research (phase I) has been given ethical approval after review by the Great Ormond Street Hospital for Children NHS Trust / Institute of Child Health Research Ethics Committee subject to the following conditions.

1. Your research must commence within twelve months of the date of this letter and ethical approval is given for a period of five months from the commencement of the project. If you wish to start the research more than twelve months from the date of this letter or extend the duration of your approval you should seek Chairman's approval.
2. You must seek Chairman's approval for proposed amendments to the research for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature, e.g. using the same procedure(s) or medicinal product(s). Each research project is reviewed separately and if there are significant changes to the research protocol, for example in response to a grant giving body's requirements you should seek confirmation of continued ethical approval.
3. Researchers are reminded that REC approval does not imply approval by the GOS Trust. Researchers should confirm with the R&D office that all necessary permissions have been obtained before proceeding.

4. It is your responsibility to notify the Committee immediately of any information which would raise questions about the safety and continued conduct of the research.
5. On completion of the research, you must submit a report of your findings to the Research Ethics Committee. You may also be required to submit annual reports.
6. Specific conditions pertaining to the approval of this project are:

- The use of the enclosed standard consent forms for the research. A copy of the signed consent form must be placed in the patient's clinical records and a copy must be kept by you with the research records.
- **The main phase of the study (phase II) will be given full ethical approval upon receipt and review of a power calculation.**

Yours sincerely

Research Ethics Coordinator

Extn 2620

Great Ormond Street Hospital for Children NHS Trust / The Institute of Child Health Local Research Ethics Committee

Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Tel: 020 7905 2620
Fax: 020 7905 2201
Email: l.howe@ich.ucl.ac.uk

29th March 2004

Reader in Clinical Pharmacology
UCL

Dear

Title: **Effects of remote ischaemic preconditioning on ischaemia reperfusion injury in the human kidney during paediatric renal transplantation**
R&D registration number: **03CC11**
Protocol number/version: **N/A**

Thank you for your email of 19th March 2004 concerning the above project. This has been received and noted by the Chairman of the GOSH/ICH Research Ethics Committee, Dr V Larcher, and I can confirm that the Chairman is not concerned about the inclusion of patients undergoing cadaveric transplantation and is happy for patients undergoing both cadaveric and live-related transplants to continue being recruited.

I look forward to receiving the power calculation for phase II of this project.

Yours sincerely,

Research Ethics Coordinator

cc. S. Loukogeorgakis, L. Rees

Great Ormond Street Hospital for Children NHS Trust / The Institute of Child Health Research Ethics Committee

Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Tel: 020 7905 2620
Fax: 020 7905 2201
Email: l.howe@ich.ucl.ac.uk

22nd April 2004

Mr S Loukogeorgakis
Vascular Physiology
ICH

Dear Mr Loukogeorgakis,

Full title of study: *Effect of remote ischaemic preconditioning on ischaemia reperfusion injury in the human kidney during paediatric renal transplantation*
REC reference number: *03CC11*

Thank you for your letter dated 31st March 2004 and the enclosed information about the power calculation for phase II of the above study. I can confirm that the power calculation is acceptable to the committee.

Membership of the Committee

The members of the Ethics Committee who reviewed the information are listed below:
(Consultant Paediatrician, Chair of the GOSH/ICH REC)
(Nurse Consultant, Haemophilia)
Professor (Professor of Medical Statistics)

Yours sincerely,

Research Ethics Coordinator

Copy to: *R&D Office, ICH*

Great Ormond Street Hospital for Children NHS Trust / The Institute of Child Health Research Ethics Committee

Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Tel: 020 7905 2620
Fax: 020 7905 2201
Email: l.howe@ich.ucl.ac.uk

22nd April 2004

Mr S Loukogeorgakis
Vascular Physiology
ICH

Dear Mr Loukogeorgakis,

Full title of study: *Effect of remote ischaemic preconditioning on ischaemia reperfusion injury in the human kidney during paediatric renal transplantation*
REC reference number: *03CC11*

Amendment number: *1; 22/04/2004*
Amendment details: *Permission to apply the same RIPC stimulus (3 cycles of 5-minute arm ischaemia and reperfusion) to both the donor and recipient 24 hours before their respective operations*

The above amendment was reviewed by the Sub-Committee of the Great Ormond Street Hospital NHS Trust/The Institute of Child Health Research Ethics Committee at the meeting held on 21st April 2004.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed below:
Dr (Consultant Paediatrician, Chair of the GOSH/ICH REC)
(Nurse Consultant, Haemophilia)

Yours sincerely,

Research Ethics Coordinator

Copy to: *R&D Office, ICH*

Central Office for Research Ethics Committees (COREC)

Room 76, B Block
40 Eastbourne Terrace
London W2 3QR

Tel: 020 7725 3431
Fax: 020 7725 3465

Website: www.corec.org.uk

Our Ref: JW/362/HT

Mr S Loukogeorgakis
Vascular Physiology
Institute of Child Health
30 Guildford Street
London WC1N 1EH

27 July 2004

Dear Mr Loukogeorgakis

Re: Institute of Child Health/Great Ormond Street Main REC Ref: 03CC11
Effects of remote ischaemic preconditioning on reperfusion injury in the human kidney during paediatric renal transplantation

I am writing to confirm that the following Committee has been appointed as the "main REC" for the study:

Name of REC: ICH/Great Ormond Street

All further communications relating to the ethical review of the study should be sent to this Committee, including notices of amendment, progress and safety reports. No further communication is required with other LRECs except where the main REC advises that further site-specific assessment is required.

Please do not hesitate to contact me if you require clarification on any of the above.

Yours sincerely

Dr ,
Director of Corporate Affairs
Central Office for Research Ethics Committees (COREC)

Great Ormond Street Hospital for Children NHS Trust / The Institute of Child Health Research Ethics Committee

Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Tel: 020 7905 2620
Fax: 020 7905 2201
Email: t.austin@ich.ucl.ac.uk

14th October 2004

Mr Stavros Loukogeorgakis
BHF Research Fellow
Vascular Physiology
ICH

Dear Mr Loukogeorgakis

Full title of study: **Effect of remote ischaemic preconditioning on ischaemia reperfusion injury in the human kidney during paediatric renal transplantation**
REC reference number: **03CC11**

The REC gave a favourable ethical opinion to this study on 30th July 2003.

Further notification(s) of no objection to the conduct of this research have been received from local site assessor(s), following site-specific assessment. The Chairman has confirmed the extension of the REC's favorable opinion to the new site(s). I attach an updated version of the site approval form, listing all sites with a favourable ethical opinion to conduct the research.

Management approval

The Chief Investigator or sponsor should inform the principal investigator at each site of the favourable opinion by sending a copy of this letter and the attached form. The research should not commence until management approval from the relevant host organisation has been confirmed at each site.

Statement of compliance (from 1 May 2004)

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Yours sincerely,

Research Ethics Coordinator

Copy: ICH/GOS R&D Office

Enclosure: Site approval form (SF1)

Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	03CC10	**Issue number:**	1	**Date of issue:**	14 October 2004
Chief Investigator:	Mr S Loukogeorgakis*				
Full title of study:	Effects of remote ischaemic preconditioning on ischaemia reperfusion injury in the human kidney				

This study was given a favourable ethical opinion by Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee on 14 October 2004. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	Post	Research site	Site assessor	Date of favourable opinion for this site	Notes [1]
Dr		(GSTT) The Guy's & St Thomas' NHS Foundation Trust St Thomas' Street London SE1 9RT	Guy's	14/10/2004	

Approved by the Chair on behalf of the REC:

..... (Signature of ~~Chair~~/Administrator*)

(**delete as applicable)*

... . (Name)

[1] *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

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Therapeutic Potential of Remote Ischaemic Preconditioning

Stavros P. Loukogeorgakis, Anna T. Panagiotidou and Raymond J. MacAllister

Centre for Clinical Pharmacology and Therapeutics, British Heart Foundation Laboratories, University College London, UK

Introduction

Ischaemia of vital tissues due to thrombosis-induced arterial occlusion is the leading cause of morbidity and mortality in the Western World and is increasing in importance in the Third World. Amongst the successful therapeutic strategies in common usage is reperfusion, either through administration of a thrombolytic agent or mechanical removal of thrombus. Timely reperfusion facilitates tissue salvage, decreasing morbidity from stroke (1) and reducing mortality following myocardial infarction (2). Nonetheless, substantial morbidity and mortality remains, despite new techniques for reperfusion and intense efforts to reduce the time between arterial occlusion and reperfusion. Therefore, adjunctive treatments to reperfusion therapy are required to further reduce tissue injury following vascular occlusion.

Mechanisms of Ischaemia Reperfusion Injury

Hypoxic cell death following interruption of the blood supply to tissues accounts for many of the pathological consequences of arterial thrombosis. Reduced oxygen supply to tissues during ischaemia prevents mitochondrial respiration, depleting cells of ATP with subsequent build up of anaerobic metabolites, reactive oxygen species and intracellular calcium, leading to cell death via necrosis or apoptosis (3). Although successful reperfusion is mandatory for tissue salvage, re-establishing blood flow initiates a cascade of events similar to an inflammatory response. This involves adhesion of circulating neutrophils to the vascular wall with subsequent tissue invasion and release of proteases, elastases and reactive oxygen species. Whilst this may be an integral part of the healing process, animal data suggests that it may also contribute to tissue injury (4). Many investigators have reported reduction in infarct size by measures that reduce the inflammatory response and have stimulated clinical trials in this area. Unfortunately, the results of such studies to limit the inflammatory response in patients with myocardial infarction have been largely negative (5;6). An entirely different approach is to limit the degree of ischaemic injury in the first place.

Ischaemic Preconditioning

Endogenous protective mechanisms against a range of tissue insults have been identified in all mammalian species studied to date. The best characterised is ischaemic preconditioning, whereby sub-lethal ischaemia induces a state of protection against subsequent prolonged ischaemia. This phenomenon has been extensively investigated in the myocardium of many species, where it can reduce infarct size by up to 75% (7). An early phase of ischaemic preconditioning ("classic" preconditioning) occurs within minutes of the preconditioning stimulus and lasts for up to 4 hours (8). The early phase of preconditioning is triggered by a number of stimuli that are generated during hypoxia, including adenosine, bradykinin, endogenous opiates and reactive oxygen species (9). These mediators act on G_i coupled cell surface receptors to initiate a cascade of second messengers, including activation of phospholipases C and D, stimulation of the ε isoform of protein kinase C (PKCε) (10), and activation of tyrosine and mitogen activated protein kinases (ERK, JNK, p38 MAPK) (11). The end effectors of preconditioning remain uncertain and include opening of mitochondrial ATP sensitive potassium (K_{ATP}) channels (12), prevention of the opening of the mitochondrial transition pore (in association with K_{ATP}

channel opening) (13), and blockade of the sodium/hydrogen exchanger (14) (**Figure 1**). It is unlikely that early preconditioning involves gene up-regulation and expression of new proteins. The fact that the preconditioned state can be achieved within minutes makes a reversible post-translational modification (phosphorylation or translocation) of pre-existing protein more likely.

A late phase of preconditioning occurs 24 hours after the preconditioning stimulus, which is more prolonged than the early phase and lasts up to 72 hours (15-17). This delayed phase of resistance to ischaemic injury has been termed second window of protection, distinguishing it from early or "classic preconditioning". The prolonged (24-hour) interval between the preconditioning event and its renewed protection one day later is consistent with new protein synthesis. The triggers for second window ischaemic preconditioning resemble early preconditioning, and similar second messengers are recruited (9). The transcriptional regulator NF-κB may well be the common pathway through which the multiple signals generated by ischaemic preconditioning initiate cardiac and other tissue gene expression (18). Activation of NF-κB leads to increased production of proteins, such as heat shock proteins (HSP 27, 70 and 72i) (19), antioxidant enzymes (superoxide dismutase; SOD) (20), cyclooxygenase (COX-2) (21) and inducible nitric oxide synthase (iNOS) (22), which may contribute to tissue protection. Opening of mitochondrial K_{ATP} channels has also been implicated in late preconditioning (23) (**Figure 2**).

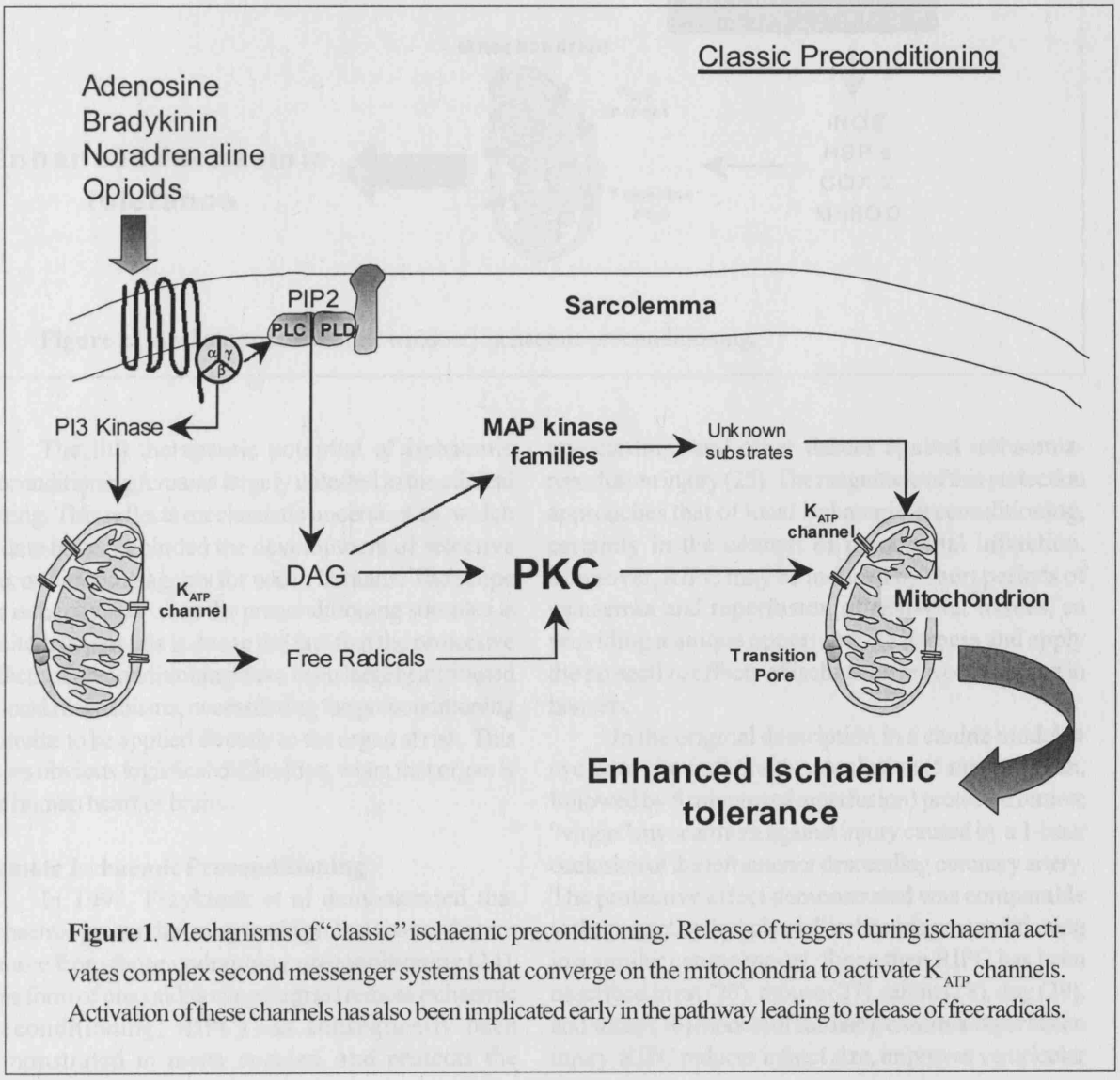

Figure 1. Mechanisms of "classic" ischaemic preconditioning. Release of triggers during ischaemia activates complex second messenger systems that converge on the mitochondria to activate K_{ATP} channels. Activation of these channels has also been implicated early in the pathway leading to release of free radicals.

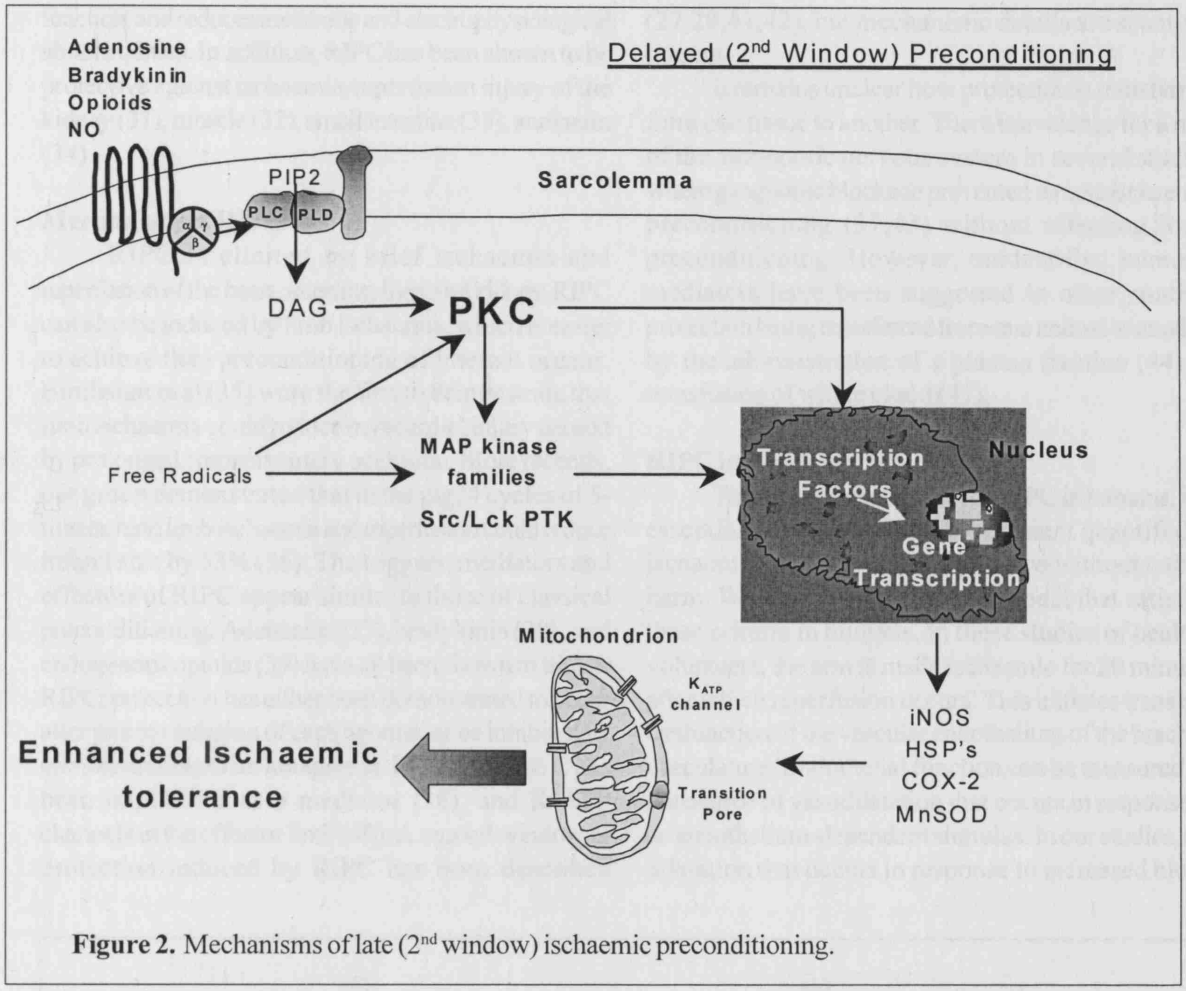

Figure 2. Mechanisms of late (2nd window) ischaemic preconditioning.

The full therapeutic potential of ischaemic preconditioning remains largely untested in the clinical setting. This reflects mechanistic uncertainties, which to date have precluded the development of selective preconditioning agents for use in humans. The scope for ischaemia *per se* as the preconditioning stimulus is limited. In part this is due to the fact that the protective effects of preconditioning have been largely attributed to local mechanisms, necessitating the preconditioning stimulus to be applied directly to the organ at risk. This poses obvious logistical difficulties, when that organ is the human heart or brain.

Remote Ischaemic Preconditioning

In 1993, Przyklenk et al demonstrated that ischaemic preconditioning could protect tissues that are remote from those undergoing preconditioning (24). This form of preconditioning (termed remote ischaemic preconditioning; RIPC) has subsequently been demonstrated in many species, and protects the myocardium and other tissues against ischaemia-reperfusion injury (25). The magnitude of this protection approaches that of local ischaemic preconditioning, certainly in the context of myocardial infarction. Moreover, RIPC may be induced by short periods of ischaemia and reperfusion of non-vital tissues, so providing a unique opportunity to harness and apply the protective effects of ischaemic preconditioning in humans.

In the original description in a canine model, 4 cycles of circumflex artery occlusion (5 minutes each, followed by 5 minutes of reperfusion) protected remote "virgin" myocardium against injury caused by a 1-hour occlusion of the left anterior descending coronary artery. The protective effect demonstrated was comparable to the protection by "classic" ischaemic preconditioning in a similar canine model. Since then RIPC has been described in rat (26), mouse (27), rabbit (28), dog (29), and sheep (30) models of cardiac ischaemia-reperfusion injury. RIPC reduces infarct size, improves ventricular

function, and reduces metabolic and electrophysiological abnormalities. In addition, RIPC has been shown to be protective against ischaemia-reperfusion injury of the kidney (31), muscle (32), small intestine (33), and brain (34).

Mechanism of RIPC

RIPC is elicited by brief ischaemia and reperfusion of the heart, intestine, liver and kidney. RIPC can also be induced by limb ischaemia, which is easier to achieve than preconditioning of internal organs. Birnbaum et al (35) were the first to demonstrate that limb ischaemia could reduce myocardial injury caused by prolonged coronary artery occlusion. More recently, our group demonstrated that in the pig, 4 cycles of 5-minute hind limb ischaemia and reperfusion could reduce infarct size by 53% (36). The triggers, mediators and effectors of RIPC appear similar to those of classical preconditioning. Adenosine (37), bradykinin (38), and endogenous opioids (39) have all been shown to trigger RIPC; protection has either been demonstrated to occur after remote infusion of each agonist, or be inhibited by administration of an antagonist. Protein kinase C has been implicated as a mediator (38), and K-ATP channels in the effector limb (40). A second window of protection induced by RIPC has been described (27;29;41;42), but mechanistic details are scanty at present.

It remains unclear how protection is transferred form one tissue to another. There is evidence for a role of the autonomic nervous system in several studies where ganglionic blockade prevented remote ischaemic preconditioning (37;43) without affecting local preconditioning. However, unidentified humoral mediators have been suggested in other studies, protection being transferred from one animal to another by the administration of a plasma fraction (44) or transfusion of whole blood (45).

RIPC in Humans

To study the mechanism of RIPC in humans, it is essential to be able to induce transient quantifiable ischaemia-reperfusion injury *in vivo* without risk of harm. We recently developed a model that satisfies these criteria in humans. In these studies of healthy volunteers, the arm is made ischaemic for 20 minutes after which reperfusion occurs. This initiates transient dysfunction of the vascular endothelium of the brachial vasculature. Endothelial function can be measured by the degree of vasodilatation that occurs in response to an endothelium-dependent stimulus. In our studies, the dilatation that occurs in response to increased blood

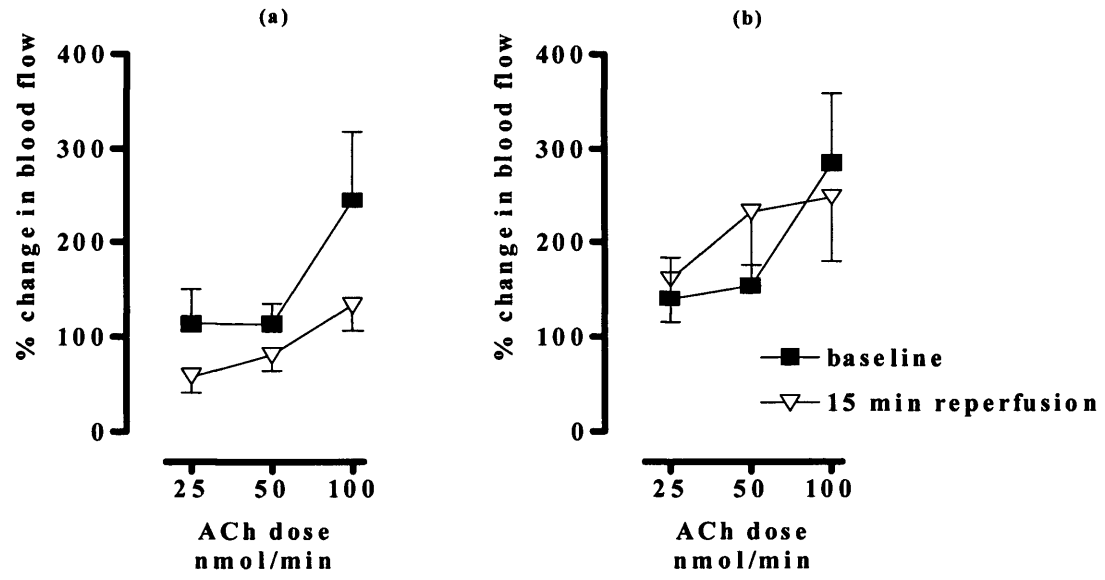

Figure 3. (a) Ischaemia reperfusion (open triangles) reduces the response of the forearm vasculature to acetylcholine consistent with endothelial dysfunction. **(b)** Remote preconditioning of the contra-lateral arm prior to ischaemia reperfusion protects the endothelium from subsequent injury.

flow, or in response to infusion of acetylcholine are both measures of endothelial function in the brachial vasculature. Ischaemia-reperfusion reduces endothelium-dependent dilatation by about 50%, and the endothelial dysfunction persists for about 60 minutes (46). RIPC is initiated by three five-minute cycles of ischaemia to the contra-lateral arm, and largely prevents endothelial dysfunction in response to ischaemia-reperfusion (36) (**Figure 3**). Using skeletal muscle magnetic resonance spectroscopy we have also shown that RIPC reduces metabolic dysfunction in muscle caused by ischaemia-reperfusion (unpublished results). These data indicate that protection caused by RIPC in humans is not limited to the vasculature. The time course of protection mirrors that described in animal models, with an early phase of protection and a second window 24 hours later, lasting for up to 48 hours (47).

Clinical Possibilities of RIPC

Although much remains to be done to understand more fully the mechanism of RIPC in humans, our current level of understanding already indicates that it may have therapeutic potential in the clinical setting. The animal data implies that it offers a similar degree of protection to classical preconditioning. RIPC also occurs in humans and is similar in time-course to that identified in animals. The stimulus is a simple risk-free procedure that can be applied in advance of planned procedures associated with ischaemia-reperfusion injury (such as cardio-pulmonary bypass grafting or organ transplantation). It may provide a window of protection 24 hours later that lasts long enough to cover the procedure and its immediate aftermath. It is even possible that for patients at risk of unpredictable ischaemia-reperfusion syndromes (such as myocardial infarction or stroke), RIPC may induce a state of ischaemic preconditioning lasting several days that could minimise tissue injury in the event of vascular occlusion. The next stage is to optimise the stimulus to elicit maximal protection and then test the therapeutic utility of RIPC in clinical trial.

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Correspondence to: Dr R. MacAllister, Centre for Clinical Pharmacology, University College London, Rayne Institute, 5 University St, London WC1E 6JJ, UK.
E-mail r.macallister@ucl.ac.uk

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Remote Ischemic Preconditioning Provides Early and Late Protection Against Endothelial Ischemia-Reperfusion Injury in Humans

Role of the Autonomic Nervous System

Stavros P. Loukogeorgakis, BSc,*‡ Anna T. Panagiotidou, BSc,*‡
Michael W. Broadhead, BSc, MRCP, FRCA,†‡ Ann Donald, AVS,*
John E. Deanfield, BA, BCHIR, MB, FRCP,* Raymond J. MacAllister, MA, MD, FRCP‡
London, United Kingdom

OBJECTIVES The aim of this study was to characterize the time course and neuronal mechanism of remote ischemic preconditioning (RIPC) of the vasculature in humans.

BACKGROUND Non-lethal ischemia of internal organs induces local (ischemic preconditioning) and systemic (RIPC) resistance to lethal ischemia-reperfusion (IR) injury. Experimental RIPC has two temporal components, is neuronally mediated, is induced by limb ischemia, and reduces infarct size. In humans, RIPC prevents IR-induced vascular injury. Determining the time course and mechanism is a prelude to clinical outcome studies of RIPC.

METHODS Endothelial IR injury was induced by arm ischemia (20 min) and reperfusion, and measured by flow-mediated dilation. To establish if there are early and late phases, RIPC (three 5-min cycles of ischemia of the contralateral arm) was applied immediately, 4, 24, and 48 h before IR. To determine neuronal involvement, trimetaphan (autonomic ganglion blocker; 1 to 6 mg/min intravenous) was infused during the application of the RIPC stimulus.

RESULTS Flow-mediated dilation was reduced by IR (8.7 ± 1.1% before IR, 4.9 ± 1.2% after IR; $p < 0.001$), but not when preceded by RIPC (8.0 ± 0.8% after IR; p = NS); RIPC did not protect after 4 h (4.9 ± 1.1% after IR; $p < 0.001$), but protected at 24 (8.7 ± 1.1% after IR; p = NS) and 48 h (8.8 ± 1.4% after IR; p = NS). Trimetaphan attenuated early (8.3 ± 1.1% before IR, 4.2 ± 0.9% after IR; $p < 0.05$) and delayed (7.3 ± 1.0% before IR, 2.3 ± 0.6% after IR, $p < 0.001$) RIPC.

CONCLUSIONS Remote ischemic preconditioning in humans has two phases of protection against endothelial IR injury; an early (short) and late (prolonged) phase, both of which are neuronally mediated. The potential for late phase RIPC to provide prolonged protection during clinical IR syndromes merits investigation. (J Am Coll Cardiol 2005;46:450–6) © 2005 by the American College of Cardiology Foundation

Ischemic preconditioning (IPC) is an innate mechanism that protects tissues from injury during ischemia and subsequent reperfusion (ischemia-reperfusion [IR] injury) (1). Preconditioning is initiated by brief, nonlethal periods of ischemia, and provides local tissue protection from the effects of further prolonged episodes of ischemia. In the heart, IPC reduces experimental infarct size by up to 75% in many different species (2–6). The effects of IPC are immediate, triggered by release of several mediators (including adenosine and bradykinin) (7,8), and dependent on the activation of complex second messenger systems (1). Immediate protection lasts just a few hours, but is followed 24 h later by a "second window" of protection (9), lasting for 48 to 72 h (10) and dependent on the induction of protective proteins (11). Although the magnitude of organ protection in experimental models of IPC is substantial, this approach has not yet led to clinical exploitation of preconditioning. This is, in part, because of the logistical difficulties of using ischemia to precondition vital organs, either in advance of clinical IR injury that is predictable (such as primary angioplasty, coronary artery bypass surgery, or transplantation), or to maintain a persistent state of preconditioning in readiness for spontaneous cardiovascular events in high-risk patients. Although much is known about the cellular mechanisms of both phases of IPC, remaining mechanistic uncertainties have so far precluded a successful pharmacologic approach to preconditioning in humans (1).

It is now clear that IPC has systemic effects that result in protection from IR injury of tissues remote from those undergoing preconditioning (12). This aspect of preconditioning, termed remote ischemic preconditioning (RIPC), was first described in the heart, where IPC initiated in the vascular distribution of one coronary artery caused protection throughout the myocardium (13). It is now apparent that protection from IPC spreads from distant organs to the heart (14,15) possibly via activation of the autonomic nervous system (14,16–18), and/or humoral factors (19,20). Mechanistically, RIPC resembles IPC and depends on similar triggers (17,18,21) and second messen-

From the *Vascular Physiology Unit, Institute of Child Health, University College London, London, United Kingdom; †Portex Anesthesia, Intensive Therapy and Respiratory Medicine Unit, Institute of Child Health, University College London, London, United Kingdom; and ‡Centre for Clinical Pharmacology and Therapeutics, University College London, London, United Kingdom. This research was supported by the British Heart Foundation.

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Abbreviations and Acronyms

FMD = flow-mediated dilation
GTN = glyceryl trinitrate
IPC = ischemic preconditioning
IR = ischemia-reperfusion
RIPC = remote ischemic preconditioning

gers (14,22,23) and in some studies provides protection for up to 24 h (24).

We have recently demonstrated, in an in vivo model of myocardial infarction, that short periods of limb ischemia induce RIPC and reduce experimental infarct size by 50% (25). In addition, using a human model of IR, we demonstrated that IPC of one arm protects the contralateral arm from endothelial IR injury, consistent with a remote preconditioning effect (25). These studies establish the principle of using limb ischemia to induce RIPC, and indicate a way in which this technique might be investigated in the clinical setting. The aim of the present study was to characterize in humans the time course of protection from RIPC and, in particular, to establish if there are two separate phases of protection. In addition, we investigated the mechanism of spread of protection to remote tissues. Defining these fundamental characteristics of human RIPC is a prerequisite for the optimal design of future clinical studies.

METHODS

Subjects. A total of 115 studies were performed on 16 healthy volunteers (12 men, 4 women; mean age ± SD 28.9 ± 7.7 years; range 21 to 48 years) who gave informed consent. Studies were approved by the local research ethics committee and performed in a temperature-controlled laboratory (24°C to 26°C). All studies repeated in the same volunteers were at least seven days apart.

Induction of IR. The nondominant forearm was made ischemic by inflating a 9-cm-wide blood pressure cuff placed around the upper arm to a pressure of 200 mm Hg for 20 min, as described previously (26).

Induction of RIPC. Remote IPC was induced by inflating a 9-cm-wide blood pressure cuff placed around the upper part of the contralateral arm. The cuff was inflated to 200 mm Hg for 5 min (ischemia), followed by a 5-min deflation. The inflation/deflation cycle was performed three times.

Assessment of conduit vessel function. Endothelial function of the brachial artery was assessed by flow-mediated dilation (FMD) of the brachial artery in the nondominant arm, as previously described (27). A B-mode scan of the brachial artery was obtained in longitudinal section between 5 and 10 cm above the antecubital fossa using a 7.0-MHz linear array transducer (spatial resolution of 0.1 mm [28]) and a standard Acuson XP10 system (Acuson, Mountain View, California). Longitudinal, electrocardiogram-gated, end-diastolic images were acquired every 3 s for offline analysis. Arterial diameter over a 1- to 2-cm segment was determined for each image with the use of automatic edge-detection software (Brachial Tools, Iowa City, Iowa). Blood flow was manipulated in the brachial artery by a 7-cm-wide blood pressure cuff placed around the forearm immediately below the antecubital fossa. After 1 min of baseline flow, the cuff was inflated to 300 mm Hg for 5 min and released, resulting in a brief episode of reactive hyperemia. Brachial artery diameter changes in response to blood flow were assessed for a further 5 min. Blood flow velocity was continuously monitored by pulsed-wave Doppler. The dilator response of the brachial artery to glyceryl trinitrate (GTN) (25 μg sublingually) was used to assess endothelium-independent dilation.

Experimental protocols. EFFECT OF IR ON VASCULAR DILATOR FUNCTION. In order to determine the effect of IR on endothelial function, FMD was assessed before ischemia and at 20 min after reperfusion (n = 13) (Fig. 1A). Similarly, the effect of IR on smooth muscle function was determined in separate studies, by assessing the dilation of the brachial artery in response to sublingual GTN (25 μg) before and after IR (n = 7) (Fig. 1B). Pilot studies indicated that GTN had a direct action to reduce IR injury if administered immediately before, but not when administered 24 h before, IR. Therefore, to assess whether IR altered the dilator response to GTN, the control dilation to GTN was measured 24 h before IR and compared with the dilation to GTN immediately after IR.

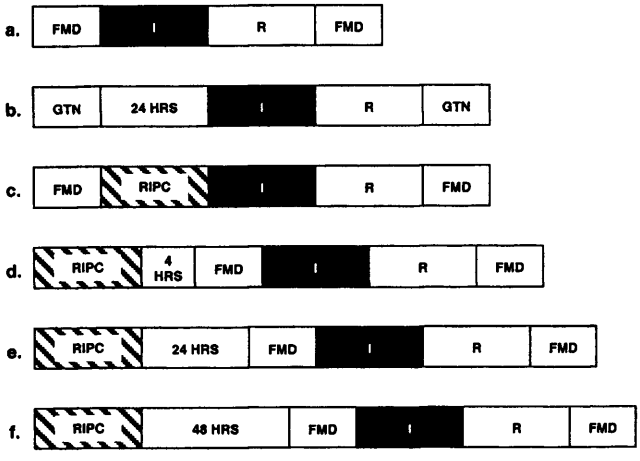

Figure 1. Protocol of studies to determine the time course of remote ischemic preconditioning (RIPC). Flow-mediated dilation (FMD) of the brachial artery was assessed before 20 min of arm ischemia (I) and at 20 min of reperfusion (R) **(a)**. The effect of IR on brachial artery smooth muscle function was determined by measuring dilation in response to sublingual glyceryl trinitrate (GTN) (25 μg) administered before and after IR **(b)**. Because pilot studies had shown that GTN prevented endothelial IR injury when administered immediately but not 24 h before, the control dilator response to GTN was determined 24 h before IR. The effect of RIPC of the contralateral arm on endothelial IR injury was determined by applying the RIPC stimulus immediately before IR **(c)**. To determine the time course of protection by RIPC, the RIPC stimulus was applied 4 h **(d)**, 24 h **(e)**, and 48 h **(f)** before IR.

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EFFECT OF RIPC ON IR INJURY TO THE ENDOTHELIUM. Flow-mediated dilation was assessed before and after IR preceded by RIPC (n = 13) (Fig. 1C). In control studies brachial FMD was measured before and after RIPC alone to determine whether RIPC had a direct effect on endothelial function (n = 7).

TIME COURSE OF THE PROTECTIVE EFFECT OF RIPC. Remote IPC was applied 4 h (n = 10) (Fig. 1D), 24 h (n = 12) (Fig. 1E), and 48 h (n = 8) (Fig. 1F) before IR; FMD was measured before and after IR.

MECHANISM OF SPREAD OF THE RIPC STIMULUS: ROLE OF THE AUTONOMIC NERVOUS SYSTEM. A venous cannula was placed in a forearm vein in the left arm under local anesthesia (1% lignocaine), and the N_N-cholinergic antagonist trimetaphan camsylate (Cambridge Laboratories, Wallsend, United Kingdom) was infused at 1 to 6 mg/min, with 1-mg/min dose increments at 5-min intervals. The dose was increased until the heart rate response to a Valsalva maneuver was abolished.

Eight volunteers underwent repeat assessment of the effects of IR alone (Fig. 1A), IR preceded by RIPC immediately (Fig. 1C), or 24 h before (Fig. 1E) on endothelial function. To determine the effect of autonomic blockade on RIPC, the same volunteers received trimetaphan by infusion during the application of the RIPC stimulus immediately before IR (Fig. 2A) or 24 h before IR (n = 7) (Fig. 2B). The effect of trimetaphan on baseline FMD (n = 4) (Fig. 2C) and the endothelial response to IR (n = 4) (Fig. 2D) was determined to exclude any direct effects on these measurements.

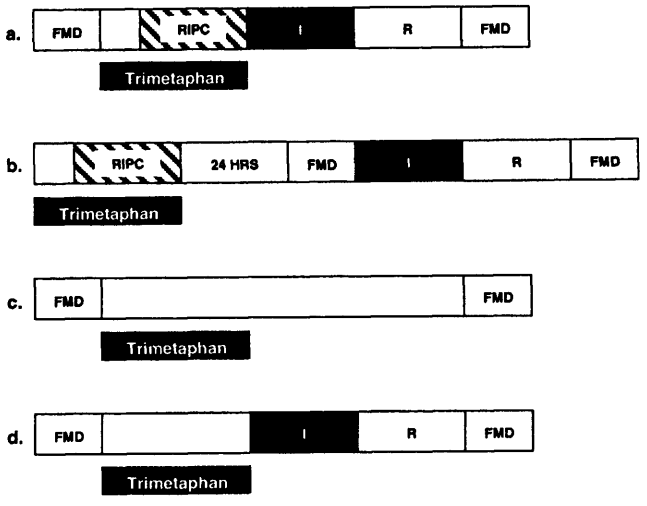

Figure 2. Protocol of studies to determine the effect of autonomic blockade on remote ischemic preconditioning (RIPC). Trimetaphan (1 to 6 mg/min) was administered by continuous intravenous infusion to cause autonomic nervous system blockade. To determine the effect of trimetaphan on early and late protection by RIPC, trimetaphan was infused during the RIPC stimulus that was applied immediately **(a)** and 24 h before ischemia and reperfusion (IR) **(b)**. Protocols **(c)** and **(d)** were designed in order to determine whether trimetaphan had direct effects on flow-mediated dilation (FMD) or the endothelial response to IR, respectively.

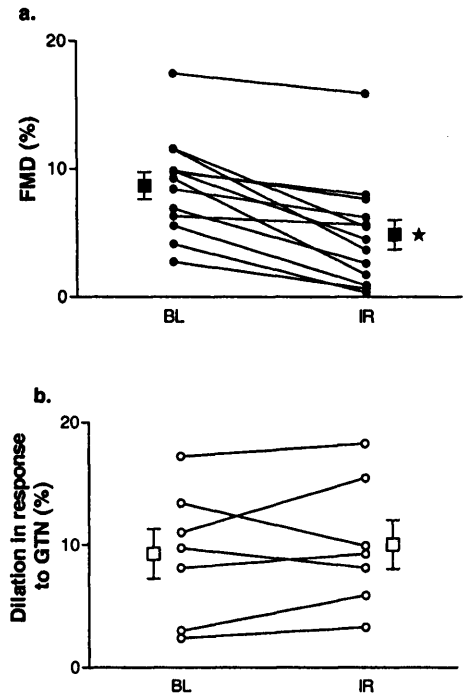

Figure 3. Effect of ischemia and reperfusion (IR) on endothelial and smooth muscle function. Flow-mediated dilation (FMD) was 8.7 ± 1.1% at baseline (BL) and was reduced by IR **(a)** (IR 4.9 ± 1.2%; *p < 0.001 vs. BL, analysis of variance; n = 13). Glyceryl trinitrate (GTN) dilation was 9.3 ± 2.0% at BL, and was unaffected by IR **(b)** (IR 10.0 ± 2.0%; p = NS, *t* test; n = 7).

Calculations and statistics. All data are expressed as mean ± SE unless otherwise stated. Brachial artery diameter was measured in millimeters and dilation expressed as percentage increase from baseline diameter. Data were compared using the Student paired *t* test or repeated measures analysis of variance (ANOVA), as appropriate. For multiple comparisons (five groups), p values by ANOVA were Bonferroni-adjusted. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

All subjects tolerated the procedures without any complications. There were no differences in the responses between men and women. The IR protocol had no effect on blood pressure, heart rate, or basal flow at 20 min of reperfusion (data not shown). Mean brachial artery diameter was 3.9 ± 0.1 mm.

Effect of IR on vascular dilator function. Ischemia-reperfusion reduced brachial artery FMD (8.7 ± 1.1% before IR vs. 4.9 ± 1.2% after IR, $p < 0.001$; n = 13) (Fig. 3A) but had no effect on blood flow during reactive hyperemia (peak to baseline volume flow ratio 8.8 ± 0.9 before IR vs. 11.2 ± 1.3 after IR, p = NS). Ischemia reperfusion had no effect on GTN dilation (9.3 ± 2.0% before IR vs. 10.0 ± 2.0%, p = NS; n = 7) (Fig. 3B).

Effect of RIPC on endothelial function. Remote IPC did not alter baseline blood flow or arterial diameter (data not

shown) and had no direct effect on brachial artery FMD (7.6 ± 0.8% before RIPC vs. 7.2 ± 0.9% after RIPC, p = NS; n = 7). Remote IPC immediately before IR prevented endothelial dysfunction (FMD 9.4 ± 0.7% before IR vs. 8.0 ± 0.8% after IR, p = NS; n = 13) Fig. 4A).

Time-course of protection by RIPC. Remote IPC did not prevent endothelial dysfunction when applied 4 h before IR (FMD 8.6 ± 1.1% before IR vs. 4.9 ± 1.1% after IR, $p < 0.001$; n = 10) (Fig. 4B). When RIPC was applied 24 h before IR, FMD was preserved (FMD 8.7 ± 1.1% before IR vs. 8.4 ± 1.2% after IR, p = NS; n = 12) (Fig. 4C). Similar findings were observed when RIPC was applied 48 h before IR (FMD 10.0 ± 0.9% before IR vs. 8.8 ± 1.4% after IR, p = NS; n = 8) (Fig. 4D).

Effect of autonomic blockade on RIPC. Autonomic blockade by trimetaphan (5.5 ± 1.3 mg/min) reduced systolic blood pressure (117.3 ± 1.9 mm Hg at baseline vs. 100.8 ± 2.1 mm Hg after autonomic blockade, $p < 0.0001$), increased heart rate (65 ± 2 beats/min at baseline vs. 89 ± 2 beats/min after autonomic blockade, $p < 0.0001$), but had no effect on diastolic blood pressure (65.4 ± 2.0 mm Hg at baseline vs. 63.9 ± 1.9 mm Hg after autonomic blockade, p = NS). The increase in heart rate in response to the Valsalva maneuver was prevented by trimetaphan (18.6 ± 1.5 beats/min increase in heart rate at baseline vs. 2.2 ± 0.8 beats/min increase in heart rate after autonomic blockade, $p < 0.0001$). The hemodynamic effects of trimetaphan were only present while it was being infused during RIPC; after cessation of the infusion, blood pressure (114.7 ± 2.4 mm Hg, p = NS vs. baseline) and heart rate (62 ± 2 beats/min, p = NS vs. baseline) had returned to normal by the time FMD was repeated.

Trimetaphan did not directly affect baseline brachial artery FMD (6.7 ± 1.3% before trimetaphan vs. 6.8 ± 0.9% after trimetaphan, p = NS; n = 4); IR reduced FMD (7.3 ± 1.2% before IR vs. 2.6 ± 0.7% after IR, $p < 0.01$; n = 8) (Fig. 5A), and this was not significantly affected by trimetaphan (FMD 8.5 ± 1.2% before IR vs. 4.5 ± 0.7% after IR, $p < 0.01$; n = 4). Remote IPC prevented endothelial dysfunction when applied immediately before IR (FMD 8.2 ± 0.9% before IR vs. 7.0 ± 0.8% after IR, p = NS; n = 8) (Fig. 5B), but protection was diminished when RIPC was applied in the presence of systemic trimetaphan (FMD 8.3 ± 1.1% before IR vs. 4.2 ± 0.9% after IR, $p < 0.05$; n = 7) (Fig. 5C). Similarly, RIPC, applied 24 h before IR, prevented IR injury (FMD 8.0 ± 1.2% before IR vs. 7.9 ± 1.6% after IR, p = NS; n = 7) (Fig. 5D), but trimetaphan blocked the protective effect of RIPC at this time point (FMD 7.3 ± 1.0% before IR vs. 2.3 ± 0.6% after IR, $p < 0.001$; n = 7) (Fig. 5E).

DISCUSSION

This study demonstrates for the first time in humans in vivo that RIPC prevents endothelial IR injury in conduit vessels with two temporally distinct phases of protection. An early

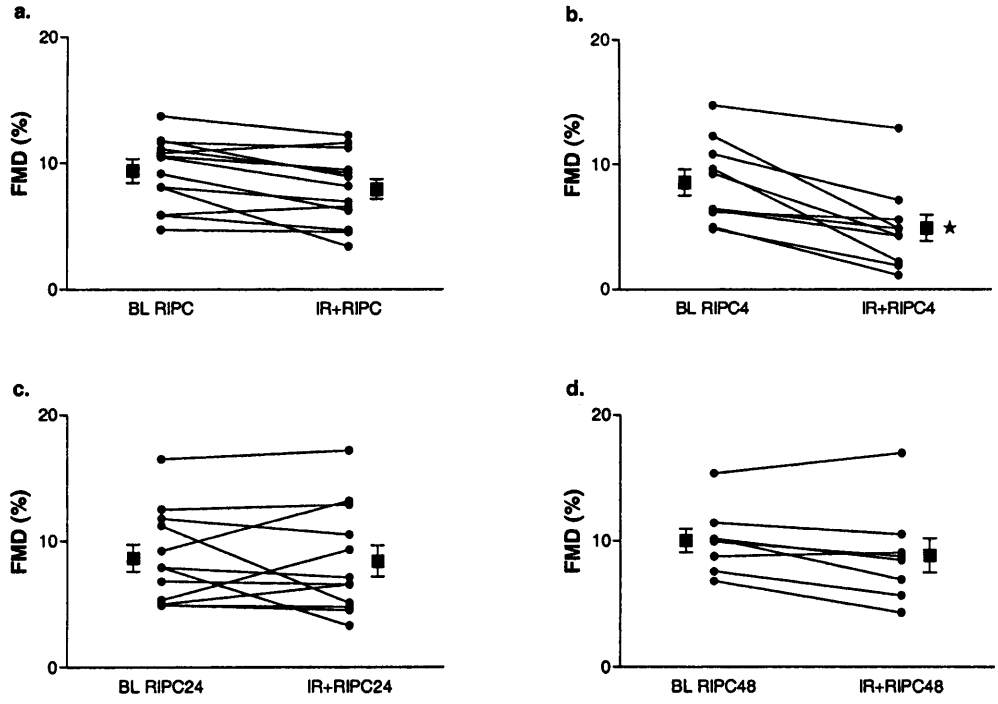

Figure 4. Time course of the protective effect of remote ischemic preconditioning (RIPC) on ischemia-reperfusion (IR)-induced endothelial dysfunction. Flow-mediated dilation (FMD) was 9.4 ± 0.7% at baseline (BL) and was unaffected by IR preceded immediately by RIPC **(a)** (IR+RIPC 8.0 ± 0.8%; p = NS vs. BL, analysis of variance [ANOVA]; n = 13). Ischemia-reperfusion reduced FMD when RIPC was applied 4 h before IR **(b)** (BL 8.6 ± 1.1% vs. IR+RIPC4 4.9 ± 1.1%; *p < 0.001, ANOVA; n = 10). However, the effect of IR to reduce FMD was prevented when RIPC was applied 24 h before IR **(c)** (BL 8.7 ± 1.1% vs. IR+RIPC24 8.4 ± 1.2%; p = NS, ANOVA; n = 12) and 48 h before IR **(d)** (BL 10.0 ± 0.9% vs. IR+RIPC48 8.8 ± 1.4%; p = NS, ANOVA; n = 8).

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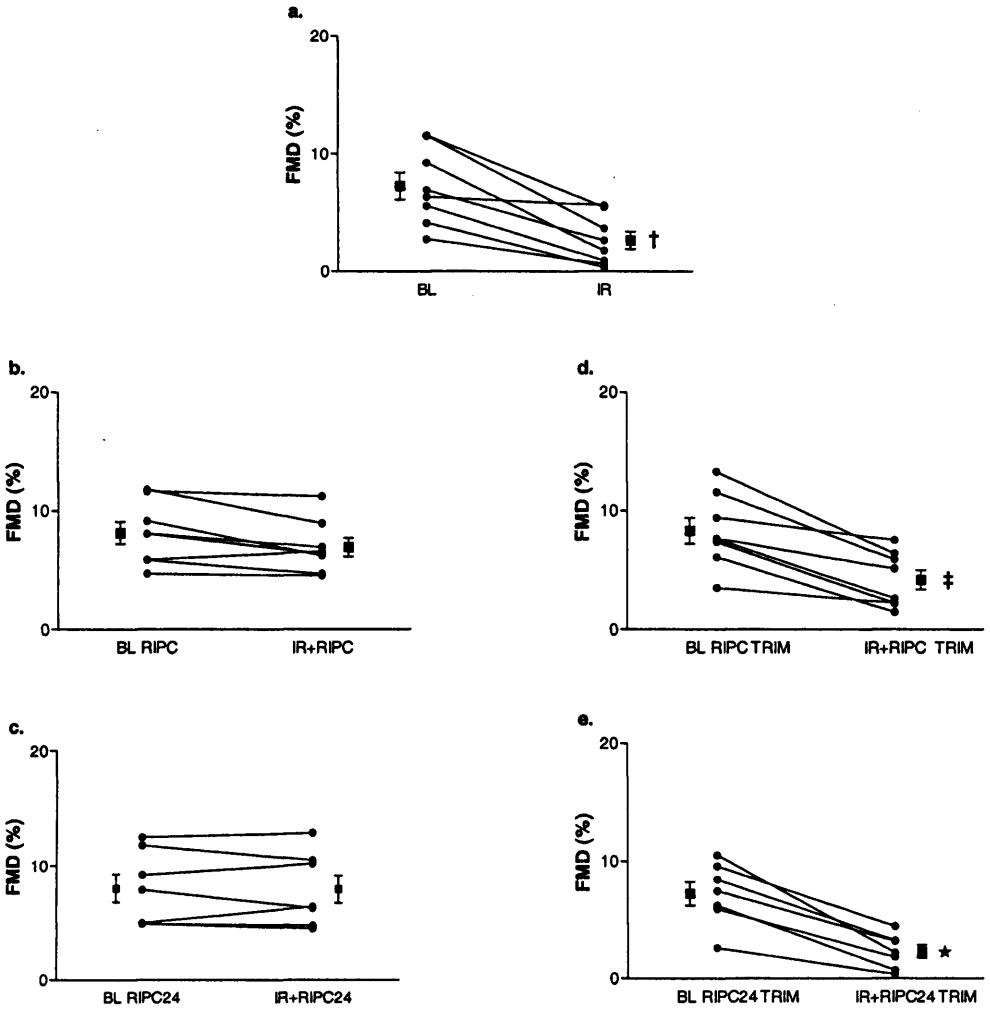

Figure 5. Effect of autonomic blockade on remote ischemic preconditioning (RIPC). Flow-mediated dilation (FMD) was 7.3 ± 1.2% at baseline (BL) and was reduced by ischemia reperfusion (IR) **(a)** (IR 2.6 ± 0.7%; †$p < 0.01$ vs. BL, analysis of variance [ANOVA]; n = 8). The effect of IR on FMD was prevented by RIPC immediately before **(b)** (BL 8.2 ± 0.9% vs. IR+RIPC 7.0 ± 0.8%; p = NS, ANOVA; n = 8) or 24 h before IR **(c)** (BL 8.0 ± 1.2% vs. IR+RIPC24 7.9 ± 1.2%; p = NS, ANOVA; n = 7). Protection by RIPC was blocked by administration of trimetaphan (TRIM) (1 to 6 mg/min intravenously) during RIPC immediately before IR **(d)** (BL 8.3 ± 1.1% vs. IR+RIPC TRIM 4.2 ± 0.9%; ‡$p < 0.05$, ANOVA; n = 7) and 24 h before IR **(e)** (BL 7.3 ± 1.0% vs. IR+RIPC24 TRIM 2.3 ± 0.6%; *$p < 0.001$, ANOVA; n = 7).

phase is activated immediately and disappears within 4 h; a second phase presents 24 h after the application of the RIPC stimulus and is sustained for at least 48 h. Both phases of RIPC are dependent on intact autonomic function. If such protection extends beyond the vasculature, then these data suggest ways in which preconditioning can be elicited in patients undergoing planned procedures complicated by IR injury.

Systemic protective effects of IPC. Ischemic preconditioning, elicited by brief ischemic episodes, reduces tissue damage occurring during prolonged ischemia (1), but the perception that it was necessary to induce IPC in the at-risk organ has limited exploitation of this phenomenon in clinical practice. The realization that preconditioning has systemic effects to induce protection in tissues remote from those undergoing preconditioning substantially increases the clinical applicability of preconditioning strategies (12). In previous work we demonstrated that RIPC could be elicited by ischemia of nonvital tissues; limb ischemia in an animal model reduced experimental myocardial infarct size and in humans prevented IR injury to the endothelium of the forearm resistance vasculature (25). Taken together these findings suggest that it may be possible to harness endogenous cardioprotection, triggered by ischemia of easily accessible tissues.

RIPC of the limb protects against endothelial IR injury. The present study demonstrates that RIPC prevents IR-induced endothelial dysfunction in conduit arteries, and is consistent with our previous data in the resistance vasculature (25). The vascular endothelium is implicated in the pathogenesis of IR injury; reduced endothelial dilator and anticoagulant function during IR injury may exacerbate vasospasm and encourage persistence of cellular aggregates and thrombus within conduit and resistance vessels. These aspects of endothelial dysfunction may directly affect the extent of tissue reperfusion (29,30), and some of the benefits

of preconditioning might be a consequence of preservation of dilator function (30), although not all studies have shown this (31,32). Changes in FMD were not explained by effects of IR injury or RIPC on blood flow increases during reactive hyperemia. Moreover, the protective effect of RIPC was not explained by changes in smooth muscle responsiveness, as RIPC did not alter the GTN-induced dilation.

Time course of protection by RIPC. Animal studies indicate that RIPC offers protection from IR injury that lasts for up to 24 h (24,33,34). However, it has not been established whether there are early and late phases of protection (as for IPC) rather than a single period of prolonged protection. Our data confirm that RIPC mirrors IPC with an early phase of protection lasting only a few hours after the RIPC stimulus, and followed 24 h later by a second window lasting for up to 48 h. The reappearance of protection and its prolonged time course is consistent with altered protein expression in the vessel wall, and it is possible that such changes may be similar to those identified in second window IPC. Further studies are needed to identify these molecular mechanisms.

Effect of autonomic blockade on RIPC. One the most intriguing questions regarding RIPC is how the transfer of the protective signal from the site of preconditioning to remote tissues occurs. There is evidence for humoral mediators that may include endogenous opioids (35,36). In addition, a neurogenic pathway has also been suggested, with evidence for involvement of the autonomic nervous system in the mechanism of the early phase (14,16–18), and sensory C fibers in the late phase (34,37,38). The present study clearly implicates the autonomic nervous system in the spread of protection during RIPC. The autonomic ganglion blocker trimetaphan was administered by intravenous infusion at a dose sufficient to cause autonomic block (confirmed by its effects on blood pressure, heart rate, and the Valsalva response). Because of its short-lived action, autonomic blockade was restricted to the RIPC phase of the protocols, and baseline hemodynamics were restored in advance of the repeat assessment of FMD. Time-control studies confirmed that trimetaphan had no direct effect on FMD, or the endothelial response to IR injury. However, when administered during RIPC, it blocked its early and late protective effects on endothelial IR injury. It is possible that release of local triggers of IPC (including bradykinin and adenosine) activate the autonomic nervous system either directly (17,18) or via sensory nerves (34,37,38), and transfers the signal to the myocardium or other remote tissues. How this leads to tissue protection is not clear at present, but animal data implicate a similar mechanism to that described for classical IPC, with activation of protein kinase C (14,22,23) and mitochondrial K_{ATP} channels (15,23,36,39). Our data do not indicate which component of autonomic function (muscarinic or adrenergic) is involved, and further studies are required to dissect these pathways.

Study limitations. In our model, IR-induced endothelial injury resolves spontaneously within 60 min of reperfusion (26), consistent with "endothelial stunning" rather than necrosis in response to 20 min of ischemia. This may explain the complete abrogation of endothelial dysfunction caused by RIPC that we observed. However, it also raises concerns that the protocol of limb RIPC that we used might be insufficient to protect from more substantial injury, as would occur in the clinical setting. We have shown in a previous study that a similar protocol of limb ischemia reduces infarct size in the pig after 40 min of coronary artery occlusion (25). Moreover, recent preliminary data in humans indicates that preconditioning using limb ischemia reduces troponin T release from the myocardium after cardiopulmonary bypass in children (40). These observations suggest that the limb is a suitable substrate to trigger systemic preconditioning, and that the present model of IR injury is suitable to examine the time course of protection.

One additional potential limitation is the lack of specificity of trimetaphan, which also has alpha-adrenoreceptor blocking properties and induces the release of histamine. Moreover, trimetaphan has direct vasodilator actions, although the mechanisms of this effect are not currently known. Although these additional actions are unlikely to account for the effects described in our study, unknown effects of the drug that alter the response of the vascular endothelium to RIPC cannot be excluded. One way of eliminating this potential source of error is to test whether RIPC can be induced in patients with autonomic nervous system dysfunction.

Conclusions. Irrespective of uncertainties regarding the details of mechanisms of IPC and RIPC, it is their similarity that is most striking, and strongly suggests that IPC and RIPC are two aspects of the same biological mechanism. When animal and human data are taken in their totality, this method of initiating IPC of vital tissues offers similar protection to that caused by direct IPC. The present study confirms that in humans RIPC offers enduring (up to 48 h) protection against endothelial IR injury. If this is applicable to other tissues, then our data suggest a simple way in which the effect of RIPC to reduce ischemic damage in clinical IR syndromes can be tested. Preconditioning could be triggered 24 h in advance of cardiopulmonary bypass surgery, angioplasty, or transplantation and provide up to 48 h of resistance to cardiac and noncardiac ischemia. Such investigations are likely to yield definitive information on the clinical utility of RIPC.

Reprint requests and correspondence: Mr. Stavros P. Loukogeorgakis, Vascular Physiology Unit, Institute of Child Health, University College London, 34 Great Ormond Street, London, WC1N 3JE, United Kingdom. E-mail: s.loukogeorgakis@ich.ucl.ac.uk.

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POSTCONDITIONING PROTECTS AGAINST ENDOTHELIAL ISCHEMIA-REPERFUSION INJURY IN THE HUMAN FOREARM

Stavros P Loukogeorgakis, Anna T Panagiotidou, Derek M Yellon, John E Deanfield, and Raymond J MacAllister

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POSTCONDITIONING PROTECTS AGAINST ENDOTHELIAL ISCHEMIA-REPERFUSION INJURY **IN THE HUMAN FOREARM**

Loukogeorgakis; Postconditioning in humans

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Stavros P. Loukogeorgakis BSc [*,‡], Anna T. Panagiotidou BSc [*,‡], Derek M. Yellon PhD DSc Hon FRCP[†], John E. Deanfield BA BChir MB FRCP [*] and Raymond J. MacAllister MA MD FRCP [‡].

[*]Vascular Physiology Unit, Institute of Child Health, University College London, [†]Hatter Cardiovascular Institute and Centre for Cardiology, University College London, [‡]Centre for Clinical Pharmacology and Therapeutics, University College London.

Correspondence to: Mr. S. Loukogeorgakis, Vascular Physiology Unit, Institute of Child Health, University College London,
34 Great Ormond Street, London, WC1N 3JE, UK.

Tel: +44 20 78138223

Fax: +44 20 78138532

Email:

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NO CONFLICT OF INTEREST

Abstract:

Background

Hypoxic cell death follows interruption of blood supply to tissues. Although successful restoration of blood flow is mandatory for salvage of ischemic tissues, reperfusion can paradoxically place tissues at risk of further injury. Brief periods of ischemia applied at the onset of reperfusion have been shown to reduce ischemia-reperfusion (IR) injury, a phenomenon called postconditioning (PostC). The aim of this study was to determine whether PostC protects against endothelial IR injury in humans, *in vivo*.

Methods and Results

Brachial artery endothelial function was assessed **using vascular ultrasound to measure flow-mediated dilation (FMD) in response to forearm reactive hyperemia. FMD was measured before and after IR (20 min of arm ischemia followed by 20 min of reperfusion)** in healthy volunteers. **To test the protective effects of PostC, 3 cycles of reperfusion followed by ischemia (each lasting 10 or 30 seconds) were applied immediately after 20 minutes of arm ischemia.** To determine whether PostC needs to be applied at the onset of reperfusion, a 1-minute period of arm reperfusion was allowed prior to the application of the 10 second PostC stimulus. **IR caused endothelial dysfunction (FMD 9.1±1.2% pre-IR, 3.6±0.7% post-IR, $p<0.001$; n=11), which was prevented by PostC applied as 10 second cycles of reperfusion/ischemia (FMD 9.9±1.7% pre-IR, 8.3±1.4% post-IR, p=NS; n=11) and 30 second cycles of reperfusion/ischemia (FMD 10.8±1.7% pre-IR, 9.5±1.5% post-IR, p=NS; n=10) immediately at the onset of reperfusion.** No protection was observed when the application of the **10 second** PostC stimulus was

delayed for 1 minute following the onset of reperfusion (FMD **9.8±1.2%** pre-IR, **4.0±0.9%** post-IR, $p<0.001$; **n=8**).

Conclusions

This study demonstrates for the first time that PostC can protect against endothelial IR injury in humans. PostC might reduce tissue injury when applied at the onset of reperfusion, by modifying the reperfusion phase of IR.

Key words: endothelium; ischemia; reperfusion injury; postconditioning

Introduction:

The prerequisite for salvage of viable myocardium and limitation of infarct size, following an acute ischemic event, is timely reperfusion (1;2). However, restoration of coronary blood flow carries with it the risk of further myocardial injury ("reperfusion injury") (3). Events occurring during reperfusion have been shown to be partly responsible for reversible (stunning) and more importantly lethal (necrosis/apoptosis) injury in animal models (4-7). Although the contribution of reperfusion injury to infarct size in humans is unknown, it is possible that minimizing reperfusion injury might reduce infarct size in humans.

Modulating blood flow on reperfusion (through gradual or intermittent reperfusion) has been shown to reduce experimental infarct size (8,9). In particular, Vinten-Johansen's group have shown that a schedule of intermittent reperfusion (termed postconditioning) applied at the onset of reperfusion reduces infarct size in animal models of myocardial IR injury (10). The protective effect of postconditioning is of a similar magnitude to that seen with ischemic preconditioning (11). However, unlike preconditioning, the influence of postconditioning is restricted to the reperfusion phase of IR injury, and this has renewed interest in the reperfusion phase as a target for cardio-protection (12).

Staat *et al* (13) have recently shown that a postconditioning protocol of intermittent reperfusion reduces myocardial injury during primary angioplasty in patients with acute myocardial infarction. We have developed a model of IR injury that results in endothelial dysfunction in conduit (14) and resistance vessels (15). In the present study, we used this model to determine the potential

protective effects of different schedules of ischemic postconditioning against endothelial IR injury in humans, *in vivo*.

Methods:

Subjects

40 studies were performed on 11 healthy volunteers (6 men, 5 women; mean age±SD 23.6±4.2 years; range, 18 to 33). All volunteers gave informed consent. Studies were approved by the local research ethics committee and performed in a temperature-controlled laboratory (24° to 26°C). All studies repeated in the same volunteers were at least 7 days apart.

Induction of Ischemia-Reperfusion (IR)

The *non-dominant* arm was made ischemic by inflating a 9-cm-wide blood pressure cuff placed around the upper part of the arm to a pressure of 200 mm Hg for 20 minutes, as described previously (14;16).

Induction of Postconditioning (PostC)

Postconditioning (PostC) was induced by applying intermittent short periods of ischemia and reperfusion on the *non-dominant* arm early during reperfusion. Following index ischemia, the arm was allowed to reperfuse for 10 seconds, after which the blood pressure cuff was inflated again to 200 mmHg, making the arm ischemic for 10 seconds. This deflation/inflation cycle was repeated for a total of 3 times (1 minute total duration; **PostC10**). **The effect of longer cycles of postconditioning (3 cycles of 30 seconds reperfusion alternating with 30 seconds of ischemia) was also determined (PostC30).**

Assessment of Conduit Vessel Endothelial Function

Endothelial function of the brachial artery was assessed by flow-mediated dilation (FMD) of the brachial artery in the non-dominant arm, as previously described (17). **In this technique, reactive hyperemia of the forearm is used to increase blood flow in the brachial artery, and this results in brachial artery dilatation.** A B-mode scan of the brachial artery was obtained in longitudinal section between 5 and 10 cm above the antecubital fossa using a 7.0-MHz linear array transducer [spatial resolution of 0.1 mm (18)] and a standard Acuson XP10 system (Acuson, Mountain View, California, USA). Longitudinal, ECG-gated, end-diastolic images were acquired every 3 seconds for offline analysis. Arterial diameter over a 1- to 2-cm segment was determined for each image with the use of automatic edge-detection software (Brachial Tools, Medical imaging Applications, Iowa City, Iowa, USA). Blood flow was manipulated in the brachial artery by a 7-cm-wide blood pressure cuff placed around the forearm immediately below the antecubital fossa. After 1 minute of baseline flow, the cuff was inflated to 300 mmHg for five minutes and released, resulting in a brief episode of reactive hyperemia. Brachial artery diameter changes in response to blood flow were assessed for a further 5 minutes. Blood flow velocity was continuously monitored by pulsed-wave Doppler.

Experimental Protocols

Effect of IR on vascular dilator function

In order to determine the effect of IR on endothelial function, FMD was assessed before ischemia (baseline) and at 20 minutes after reperfusion (n=11; Figure 1, protocol a). We have previously demonstrated that this protocol results in brachial

artery endothelial dysfunction but does not have an effect on vascular smooth muscle function, as it did not alter the dilator response to glyceryl trinitrate (GTN) (14).

Effect of PostC on endothelial IR injury

In order to establish that protection against endothelial IR can be achieved by modifying reperfusion, PostC was induced, in the same group of volunteers. 3 cycles of 10 seconds of reperfusion and 10 seconds of ischemia (PostC10; n=11; figure 1, protocol b) or 3 cycles of 30 seconds of reperfusion and 30 seconds of ischemia (PostC30; n=10; figure 1 protocol c) were applied *immediately* following index ischemia. To determine whether PostC needs to be applied at the onset of reperfusion, a 1-minute period of arm reperfusion was allowed prior to the application of the 10-second cycle PostC stimulus (*Delayed*PostC10; n=8; figure 1, protocol d).

Calculations and Statistics

All data are expressed as mean±SE unless otherwise stated. Brachial artery diameter was measured in millimeters and dilation expressed as percentage increase from baseline diameter. The FMD flow stimulus during reactive hyperemia was expressed as the ratio of peak to baseline volume-flow per minute. Data were compared using paired student's *t*-test, or repeated measures ANOVA as appropriate. For multiple comparisons (**8** groups), p values by ANOVA were Bonferroni adjusted. In all cases, $P<0.05$ was considered statistically significant.

Results:

All subjects tolerated the procedures without any complications. There were no differences in the responses between men and women.

Effect of IR on vascular dilator function

The IR protocol had no effect on blood pressure and heart rate (table 1, **columns A and B**). IR reduced brachial artery FMD (9.1±1.2% pre- versus 3.6±0.7% post-IR, p<0.001, ANOVA; n=11; figure 2a), which is consistent with endothelial dysfunction. These results could not be explained by differences in brachial artery diameter (3.4±0.2mm pre- versus 3.4±0.2mm post-IR, p=NS, *t*-test) and FMD flow stimulus (peak to baseline volume-flow ratio 11.3±1.5 pre- versus 10.9±1.0 post-IR, p=NS, *t*-test) (table 1, **columns A and B**).

Effect of PostC on endothelial IR injury

PostC had no effect on blood pressure, heart rate, arterial diameter and flow stimulus during reactive hyperemia (table 1, columns C to H). 10 second cycle PostC (PostC10), applied immediately at the onset of reperfusion prevented IR-induced endothelial dysfunction (FMD 9.9±1.7% pre- versus 8.3±1.4% post-IR+PostC10, p=NS, ANOVA; n=11, figure 2b). Similarly, 30 second cycle PostC (PostC30), was also protective (FMD 10.8±1.7% pre- versus 9.5±1.5% post-IR+PostC30, p=NS, ANOVA; n=10, figure 2c). However, no protection was observed when the application of the **PostC10** stimulus was delayed for 1 minute following blood flow restoration to the arm (FMD **9.8±1.2%** pre- versus **4.0±0.9%** post-IR+*Delayed*PostC**10**, p<0.001, ANOVA; n=**8**; figure 2**d**). Mean post-IR FMD in PostC**10 and PostC30** studies was significantly higher compared to respective values

obtained in control **(IR alone; $p<0.001$, ANOVA)** and *Delayed*PostC**10** experiments (**$p<0.001$**, ANOVA) **(table 1)**.

Discussion:

This study demonstrates for the first time in humans *in vivo*, that ischemic postconditioning prevents endothelial ischemia-reperfusion (IR) injury in conduit vessels. The first moments of reperfusion are critical for **endothelial** protection by postconditioning, as the protective effect disappeared when the stimulus was applied one minute following the onset of reperfusion.

Ischemic postconditioning describes protection against IR injury by a series of brief interruptions of blood flow applied at the onset of reperfusion. Studies by Zhao et al (11) and Halkos et al (19) demonstrated in a canine model of myocardial infarction that the application of a postconditioning protocol immediately following coronary artery occlusion, reduced myocardial infarct size, an effect comparable to that of ischemic preconditioning (IPC). Since then the cardio-protective effects of postconditioning have been confirmed in the *in vivo* and *ex-vivo* heart in rats (20;21) and rabbits (22;23) as well as in isolated rat cardiomyocytes (24). More recently, Staat et al have demonstrated that postconditioning reduces infarct size in patients undergoing primary angioplasty for acute myocardial infarction (13).

In the present study, we used arm ischemia to model IR injury in humans. Previously we have shown that ischemia causes transient endothelial dysfunction of the brachial artery, with preservation of smooth muscle function (14). This model is a convenient way to compare different schedules of postconditioning stimuli. In our IR model, the degree of protection by postconditioning is similar to that achieved by IPC and remote IPC (RIPC) in previous studies in healthy

volunteers (14;16). This is in agreement with animal data (19;21) and suggests that injury suffered during reperfusion is largely responsible for the IR-induced endothelial dysfunction we observed. Moreover, our present findings demonstrate that postconditioning induces protection against endothelial IR injury by modifying events occurring during the early stages of reperfusion (20); a delay in the application of the postconditioning stimulus for as little as one minute resulted in loss of protection. Cycle length (10 versus 30 seconds) mattered less than the timing of the initiation of the postconditioning schedule. In this regard it is worth noting that the recent study of primary angioplasty in patients utilized a postconditioning protocol of four 60 second cycles of ischaemia/reperfusion, also initiated within the first minute of reperfusion (13). It remains to be determined to what extent these results in the brachial vasculature can be extrapolated to the coronary vasculature. Nonetheless, the present study provides corroborative evidence that postconditioning occurs in humans, and has similar characteristics to that described in animal models and patients with acute coronary disease.

Postconditioning was initially proposed to act by reducing neutrophils-mediated damage in post-ischemic myocardium (11;20) but this cannot be the sole mechanism of action as it is protective in isolated perfused hearts (21;23) and cell culture systems (24) that are neutrophil-free. There may be effects to reduce reactive oxygen species (ROS) production (19;20;25) and calcium overload (24). Moreover, emerging evidence indicates that protection may be dependent on adenosine receptor stimulation (23), opening of mitochondrial K_{ATP} channels (22), activation of the pro-survival kinases PI3K-Akt (21;23;26) and Erk1/2 (22) and inhibition of mitochondrial

permeability transition pore (mPTP) opening (27), key factors implicated in IPC-induced protection. These observations suggest that postconditioning and IPC have common signaling pathways, which may explain why these protective phenomena are equally effective in protecting against IR injury (11;19;21).

In humans, preservation of FMD by postconditioning is consistent with increased NO availability during reperfusion, either due to decreased NO inactivation (11;24) and/or increased NO synthesis (21). In our model, IR-induced endothelial injury resolves spontaneously within 60 minutes of reperfusion (16), consistent with "endothelial stunning" rather than necrosis in response to 20 minutes of ischemia. This may explain the complete abrogation of endothelial dysfunction caused by postconditioning that we observed. **Whether the postconditioning schedule we used will protect from more substantial endothelial injury (as would occur in the clinical setting of acute myocardial infarction), or be protective in patients with pre-existing endothelial dysfunction (as occurs in patients with risk factors for atherosclerosis) remains to be determined.**

In summary, our data demonstrate an effect of postconditioning to prevent endothelial IR injury in the human forearm vasculature. The data indicate that much of the endothelial dysfunction caused by sub-lethal IR injury occurs during reperfusion. Moreover, mechanisms activated in the immediate phase of reperfusion are necessary for endothelial IR injury. These data support the idea that the reperfusion phase of IR injury is a legitimate therapeutic target. **Given the increasing role of primary intervention in the treatment of acute coronary syndromes, postconditioning**

schedules or pharmacological manipulation may modify reperfusion injury, and reduce infarct size sufficiently to improve outcomes (28).

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Figure/Table legends:

Table 1. Summary of pre- and post-IR data from sequential studies in healthy volunteers

Brachial artery endothelial function was assessed by FMD before and after IR alone *(columns A and B)*, IR+PostC**10** ***(columns C and D)***, **IR+PostC30** ***(columns E and F)*** or IR+*Delayed*PostC**10** (PostC10 applied 1 minute after the onset of reperfusion) ***(columns G and H)***. SBP/DBP: systolic/diastolic blood pressure; HR: heart rate **(in beats per minute; bpm)**. Flow stimulus during reactive hyperemia was expressed as the ratio of peak to baseline volume flow (no units). Flow mediated dilatation (FMD) was expressed as peak % dilation from baseline brachial artery diameter. **** p<0.001 FMD (A) vs. (B) and (G) vs. (H); † p<0.001 FMD (D) vs. (B) and (F) vs. (B); ‡ p<0.001 FMD (D) vs. (H) and (F) vs. (H) (repeated measures ANOVA).***

Figure 1. Protocol of studies to determine the protective effects of PostC against endothelial IR injury

Flow-mediated dilation (FMD) of the brachial artery was assessed before 20 minutes **(min)** of arm ischemia (I) and at 20 minutes of reperfusion (R) (a). To determine whether modification of reperfusion by postconditioning (PostC) can result in protection against endothelial IR injury, 3 cycles of 10 seconds (sec) arm reperfusion and 10 seconds ischemia (PostC**10**; b) **or 3 cycles of 30 seconds of reperfusion and 30 seconds of ischemia (PostC30; c)** were applied immediately following restoration of blood supply to the arm. In order to establish if there is a requirement for PostC to be applied at the onset of reperfusion, the PostC10 stimulus was administered following 1 minute of arm reperfusion (*Delayed*PostC**10; d**).

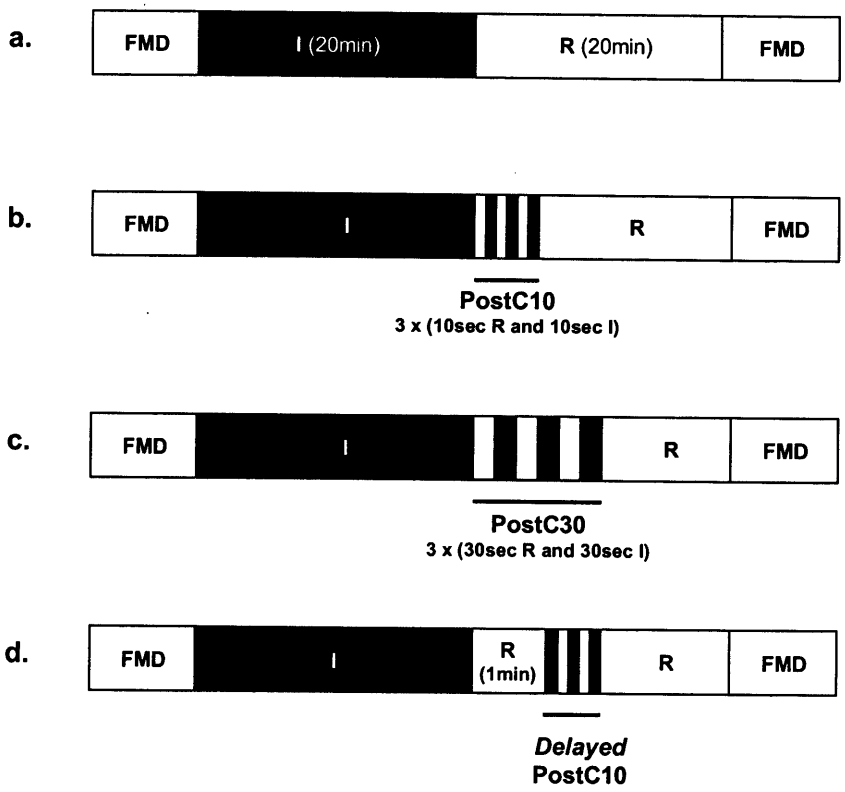
Figure 2. Effect of PostC on endothelial IR injury

FMD was 9.1±1.2% at baseline (BL) and was reduced by IR (a; IR 3.6±0.7%; *p<0.001, ANOVA; n=11). Modification of reperfusion by 3 cycles of 10 seconds reperfusion and 10 seconds ischemia (PostC**10**), applied immediately at the onset of reperfusion, prevented the reduction in FMD (b; BL 9.9±1.7% vs. IR+PostC10 8.3±1.4%; p=NS, ANOVA; n=11). **A PostC protocol consisting of 3 cycles of 30 seconds reperfusion and 30 seconds ischemia (PostC30) was equally effective in protecting against endothelial IR injury (c; BL 10.8±1.7% vs. IR+PostC30 9.5±1.5%; p=NS, ANOVA; n=10).** PostC needs to be applied at the onset of reperfusion, as no protection was observed when PostC10 was induced after 1 minute of reperfusion (*Delayed*PostC10) (**d**; BL **9.8±1.2%** vs. IR+*Delayed*PostC**10 4.0±0.9%**; *p<0.001, ANOVA; n=**8**).

Table 1.

	IR alone (n=11)		IR+PostC10 (n=11)		IR+PostC30 (n=10)		IR+DelayedPostC10 (n=8)	
	Pre (A)	*Post (B)*	*Pre (C)*	*Post (D)*	*Pre (E)*	*Post (F)*	*Pre (G)*	*Post (H)*
SBP (mmHg)	112±2	112±2	114±2	114±2	113±3	115±3	112±2	111±3
DBP (mmHg)	62±1	64±2	65±2	64±2	63±2	65±2	65±3	63±3
HR (bpm)	63±2	60±3	68±3	66±3	68±4	67±4	65±3	63±3
Baseline arterial diameter (mm)	3.4±0.2	3.4±0.2	3.2±0.2	3.2±0.2	3.0±0.2	3.1±0.2	3.3±0.2	3.3±0.3
Flow stimulus	11.3±1.5	10.9±1.0	8.3±1.7	8.5±1.7	8.6±1.4	7.8±1.3	8.0±1.8	8.1±1.4
FMD (% increase in diameter)	**9.1±1.2**	**3.6±0.7** *	**9.9±1.7**	**8.3±1.4** †‡	**10.8±1.7**	**9.5±1.5** †‡	**9.8±1.2**	**4.0±0.9** *

Figure 1.


a.
FMD
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R (20min)
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b.
FMD
I
R
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PostC10
3 x (10sec R and 10sec I)
c.
FMD
I
R
FMD
PostC30
3 x (30sec R and 30sec I)
d.
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I
R
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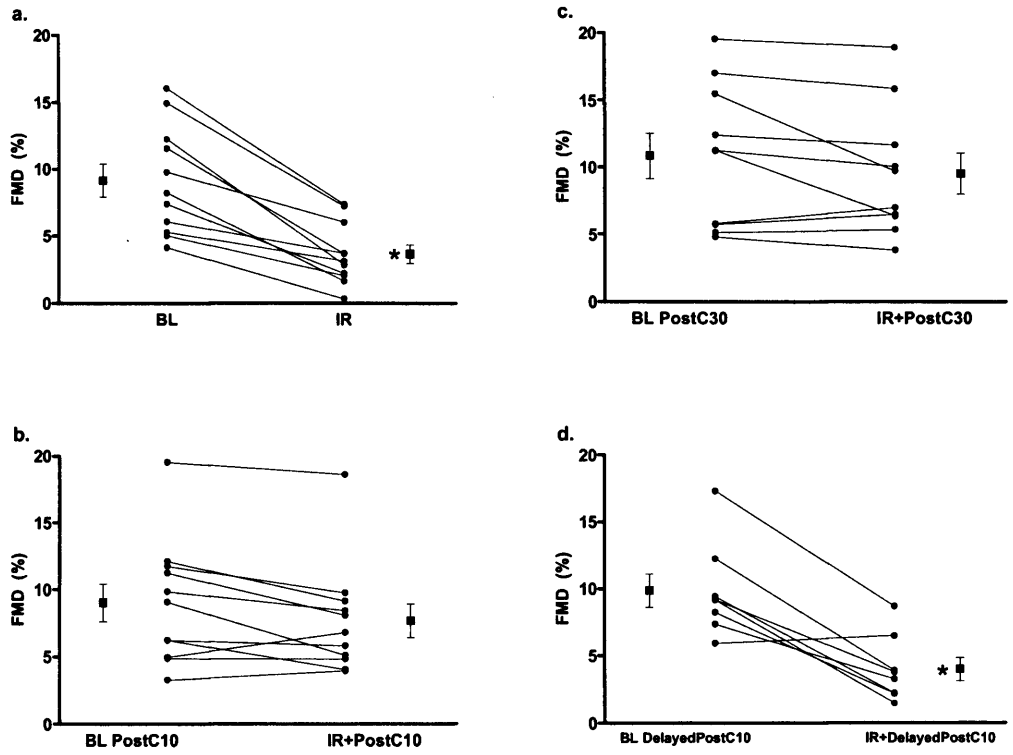
Delayed
PostC10

Figure 2.


a.
FMD (%)
20
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b.
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